The use of reverse transcription polymerase chain reaction to identify metastases in sentinel lymph nodes of patients with breast cancer

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Contents

Abstract

Over the past decade the most exciting surgical advance in the management of breast cancer has been the introduction of sentinel lymph node biopsy. The American Society of Clinical Oncology (ASCO) published guidelines on the use of sentinel lymph node biopsy in 2005 suggesting that it was a safe and accurate way to stage the axilla in patients with early breast cancer and who were clinically node negative and therefore an appropriate alternative to axillary node clearance in these patients (Lyman et al. 2005). Sentinel node biopsy has now become the standard of care in many centres worldwide however in the United Kingdom only a minority of breast surgeons are currently performing the technique.

In addition to providing a less morbid procedure for the patient, sentinel node biopsy can increase the detection of metastatic disease leading to a potential 'stage shift'. The technique offers the opportunity to detect smaller disease foci in the sentinel node because one or a few sentinel nodes can be more intensively examined than an entire clearance specimen. There is some evidence that micrometastases diagnosed by serial sectioning or immunohistochemistry lead to poorer survival rates however there remains some controversy about this and they are not as yet a required part of sentinel lymph node evaluation as laid down in the ASCO guidelines. Molecular techniques such as RT PCR can identify isolated tumour cells although the clinical significance of these is unknown.

The aim of this study was to use the reverse transcription polymerase chain reaction (RT PCR) to assess sentinel lymph nodes from 2 groups of breast cancer patients. Group 1 consisted of 54 patients from whom 68 half sentinel nodes had been obtained while group 2 consisted of 50 patients from whom 60 sentinel nodes were obtained. Three putative breast cancer antigens or 'markers' were identified in the sentinel nodes, cytokeratin 19 (CK 19), mucin 1 (MUC 1) and carcinoembryonic antigen (CEA). The expression rates of these markers were compared with each other and with the conventional H&E histology of these nodes. In particular the technique was used and compared in the 2 groups where half of each sentinel node was examined by RT PCR in group 1 and a 4μm

section was cut from each sentinel node and examined by RT PCR in the sentinel nodes from group 2. There are no reports in the literature where RT PCR has been used to evaluate different amounts of sentinel lymph node tissue.

The initial hypotheses of this study were first that the expression rates of the 3 markers, as determined by RT PCR, would correlate more closely with conventional histology than would the expression of any individual marker. Second, this correlation would be closer when more of the sentinel node, ie half a node compared to a 4μm section, was processed for RT PCR. Third, the number of histologically negative sentinel lymph nodes upstaged by RT PCR would therefore be greater when half nodes were processed.

RNA extraction and subsequent RT PCR was performed on one half of each of 68 sentinel nodes and from a single 4μm section cut from each of 60 sentinel nodes.

The sentinel nodes harvested from the patients in group 1 were performed using radioactive colloid in 56% of cases and blue dye in all cases. In group 2, a total of 65 procedures were carried out and a sentinel node was identified in 50 patients, giving a detection rate of 77%. The sentinel node biopsies performed in group 2 were all done using a combination of radioactive colloid and blue dye. A pre-operative scan was performed in all 65 cases and was positive in only 19 of these (29.2%). The combined false negative rate for both groups, based on H&E histology, was 25.7% (19/65 patients).

The results showed that there was poor correlation both between the marker expression and H&E histology and between the different markers themselves in individual sentinel nodes in both groups 1 and 2. Disappointingly, only one third or less of the nodes from each group displayed the same expression patterns for all 3 markers. Contrary to what had been expected, the expression rate did not seem to depend on the proportion of the node that was examined by RT PCR. More nodes however were upstaged when half the node was examined by RT PCR rather than when only a 4μm section was examined.

While the association of marker expression with H&E histology was poor, a strong association between CK 19 expression and Nottingham Prognostic Index (NPI) was found in both groups of patients. This suggests that both the size and grade of the tumour as well as extent of lymph node involvement may contribute to the likelihood of CK 19 expression in the sentinel node. There was no such association for MUC 1 or CEA.

In conclusion, even when the expression rates of CK 19, MUC 1 and CEA were considered together and compared to H&E histology, RT PCR was not sufficiently sensitive to reliably detect metastatic disease in sentinel nodes in these breast cancer patients. This was the case even when half the sentinel node was examined by RT PCR. The technique is therefore not safe for use in clinical practice when applied to these three markers. However CK 19 expression by the sentinel nodes from both groups of patients was found to be significantly associated with NPI and therefore this marker would be worthy of further study.

Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for or is currently being submitted in candidature for another degree.

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Abbreviations

axillary recurrence rate	(ARR)
beta mercaptoethanol	(βME)
base pair	(bp)
cytokeratin 19	(CK 19)
carcinoembryonic antigen	(CEA)
complimentary deoxyribonucleic acid	(cDNA)
detection rate	DR
deoxyribonucleic acid	(DNA)
disease free survival	(DFS)
diethylpyrocarbonate	(DEPC)
deoxynucleotide triphosphates	(dNTPs)
false negative rate	(FNR)
haematoxylin and eosin	(H&E)
immunohistochemistry	(IHC)
isolated tumour cells	(ITC)
mega Bequerel	(MBq)
mucin 1	(MUC 1)
mean follow up	(MFU)
micrometastases	(MM)
magnesium chloride	($MgCl_2$)
sodium ethylenediamine tetraacetic acid	(Na_2EDTA)
sodium hydroxide	(NaOH)
non sentinel lymph node	(NSLN)
Nottingham prognostic index	(NPI)
polymerase chain reaction	(PCR)
reverse transcription polymerase chain reaction	(RT PCR)
ribonucleic acid	(RNA)
ribonuclease	(RNase)

serial sectioning	**(SS)**
sentinel lymph node	**(SLN)**
tris acetic acid EDTA	**(TAE)**

Hypotheses and Aims

Over the last 10 years sentinel node biopsy has emerged as an alternative to axillary sampling or clearance for staging the axilla in breast cancer patients although clearly the technique is still very much in its infancy. Many studies have now been published achieving detection rates of over 90% and false negative rates of less than 5% and negligible axillary recurrence rates have been seen in those studies where no other axillary treatment has been given in the event of a negative sentinel node. The long term follow up results of large multi centre phase III trials will not be available for some time and there are many unanswered questions regarding the technique.

Perhaps the most controversial issues surrounding sentinel node biopsy are first how best to analyse the sentinel node and second what size of sentinel node metastasis should be regarded as clinically significant. Disease in the sentinel node detected by conventional histology and techniques such as serial sectioning and immunohistochemistry are really surrogates for macrometastases and micrometastases respectively. There is some evidence that micrometastases are clinically significant although this point is controversial.

Molecular techniques such as RT-PCR can identify isolated tumour cells. This technique has been used particularly to look at bone marrow and blood samples from breast cancer patients in order to identify metastases however relatively few studies have used the technique to look at sentinel lymph nodes. As yet there has been no marker identified to be 100% sensitive and 100% specific in the diagnosis of metastatic breast cancer. Three of the most widely applied putative markers are cytokeratin 19 (CK 19), mucin 1 (MUC 1) and carcinoembryonic antigen (CEA). Both CK 19 and MUC 1 are expressed by at least 90% of breast tumours while CEA is a less sensitive marker, expressed in around 70% of breast tumours. Due to the nature of the technique, any tissue evaluated in this way is not available to the histopathologist and so an obvious dilemma is how much of the sentinel node should be processed for RT PCR. There are as yet no published studies using RT PCR to detect breast cancer markers in different amounts of sentinel lymph node tissue.

Based upon the available evidence to date the following hypotheses are proposed:

1. The expression of a panel of 3 breast cancer markers by sentinel lymph nodes from breast cancer patients as determined by RT PCR will correlate more accurately with histological findings than when expression of only a single marker is considered.
2. The correlation between marker expression by RT PCR and conventional histology will be higher when a greater proportion of the sentinel lymph node is processed for RT PCR.
3. The number of histologically 'negative' sentinel nodes upstaged by RT PCR will again be greater when a greater proportion of the sentinel nodes have been examined by RT PCR.

The aims of this thesis were therefore to establish in sentinel nodes from breast cancer patients:

1. The correlation between expression rates of CK 19, MUC 1 and CEA with each other and with H&E histology
2. The expression rates of the three markers in half sentinel nodes (group 1) versus 4μm sections cut from sentinel nodes (group 2)
3. The percentage of histologically negative sentinel nodes upstaged by RT PCR for the three markers in the two groups of sentinel nodes (groups 1 and 2)

Chapter 1
Literature Review

1.1. General introduction

Breast cancer is the most common cancer in women in the UK. It represents 30% of all female cancers. Almost 41 000 women are diagnosed with the disease in the UK each year and every month it accounts for over 1 000 deaths. On a positive note however, deaths from breast cancer have reduced by over 20% in the past 10 years due to improved adjuvant therapy together with screening and earlier presentation of symptomatic disease.

Current surgical management of breast cancer in most centres involves resection of the primary tumour either by mastectomy or wide local excision together with axillary node dissection. The past 100 years or so have seen many changes in surgical management with a general trend towards less aggressive resection.

In 1907 Halsted published a report on radical mastectomy for the treatment of breast cancer (Halsted, 1907). The next few decades saw more extensive procedures such as the extended radical mastectomy and the super-radical mastectomy. A review published in 1932 however suggested that although these procedures usually resulted in adequate local control, long term survival did not improve with more radical resections (Lewis et al. 1932). In 1948 Patey and Dyson described the modified radical mastectomy with preservation of pectoralis major (Patey et al. 1948) and since then more conservative procedures have in general prevailed.

The introduction of adjuvant radiotherapy to the breast and chest wall has also been a significant factor in the move to less radical surgery, allowing conservation of the breast where possible. Randomised trials beginning in the late 1960s demonstrated that the combination of local surgery and radiotherapy could achieve results similar to those of the radical and modified radical mastectomy (Veronesi et al. 1981, Fisher et al. 1985, Fisher et al. 1989) and more recent trials such as the EORTC 10801 trial have confirmed these results (Van Dougan et al. 1992).

Experiments in the 1950s and 60s by Fisher showed that particles injected into breast lymphatics rapidly appeared in the venous system, demonstrating venous-lymphatic communications (Fisher et al. 1966) and therefore the possibility of blood-borne metastases. His hypothesis suggested that the long term cure of breast cancer was therefore dependent on systemic treatment as well as resection of the primary tumour. Since then there have been many advances in adjuvant endocrine therapy and chemotherapy with improved survival rates.

1.2. The lymphatic system in breast cancer

1.2.1. Basic anatomy of the lymphatic system

In the 17th century cadaveric and animal studies lead to the identification of the lymphatic system and an atlas demonstrating the lymphatics of the entire human body was published in 1787 by Paolo Mascagni. Lymphatic channels were later linked to regional nodes.

The basic units of the lymphatic system are semi permeable lymph capillaries composed of a single layer of endothelium and at this level the movement of cells and fluid is by simple diffusion. These drain into one way collecting lymph vessels which travel alongside veins to regional lymph nodes. Lymph trunks exit the nodes and drain into terminal vessels. All organs apart from the central nervous system, spleen, liver lobules, muscle and bone marrow contain lymphatics. There is communication between the lymphatic and venous systems at the pre and post nodal level and so cells and antigens can bypass regional nodes by this route.

1.2.2. The axilla and breast cancer

In 1849 Virchow postulated that lymph nodes were a physical barrier to the spread of cancer (Virchow, 1849) and over the next few decades theories about cancer spread

developed (Stiles, 1892). These theories contributed to the evolving surgical management of breast cancer such as Halsted's radical mastectomy in which the ipsilateral axillary lymph nodes were excised en bloc (Halsted, 1907). This did not however improve the survival rate compared to that of his predecessors who did not perform axillary dissection. More radical lymphadenectomies have also been attempted such as excision of the internal mammary and supraclavicular nodes but these did not improve survival and were at the expense of significant morbidity (Urban et al. 1971). An axillary dissection to some extent has however remained an integral part of the surgical management of breast cancer over the past century.

Each quadrant of the breast is dominantly drained by 1 or 2 collecting lymphatic vessels. The quadrants are also interconnected via the subareolar plexus as confirmed by one recent cadaver study (Pavlista et al 2005). Within the axilla, 3 levels of lymph nodes are recognised and are described according to their relation to pectoralis minor. Level I, II and III nodes are situated lateral, posterior and medial to pectoralis minor respectively. Disease spread within the axilla is usually sequential from level I through to III however 'skip metastases' occur when disease is found at a higher level but lower levels appear to be disease free. Most of the studies which have looked at the pattern of axillary lymph node metastases have found a skip metastases rate of less than 4% of all node positive patients (Veronesi et al. 1971, Rosen et al. 1983, Van Lancker et al. 1995). Gaglia et al however found skip metastases in 14.9% of 492 patients and they found that skip metastases were more common when less than 4 nodes were tumour positive (Gaglia et al. 1987).

As well as providing local control and removing tumour burden, axillary dissection provides essential staging information. The overall incidence of nodal disease is 35-40% and has remained unchanged over the last few decades. Lymph node status is the single most important prognostic factor in breast cancer (Dent, 1996). The presence of axillary node metastases decreases 5 year survival by 40% in patients with early breast cancer (Nemoto et al. 1980, Carter et al. 1989). The number of involved nodes also has prognostic significance (Nemoto et al 1980). Other important prognostic factors include

the size of the primary tumour, grade, presence of lymphovascular invasion, oestrogen receptor status and HER 2 status but none is as reliably correlated with outcome as axillary node status. The Nottingham Prognostic Index (NPI) is an index derived from a retrospective multivariate analysis based on lymph node stage, grade and tumour size stratifying patients into 3 prognostic groups. It was first described in 1993 (Galea et al. 1993) and predicted 15 year survival rates for groups 1, 2 and 3 as 80%, 42% and 13% respectively. Since then NPI has been widely applied and verified (Balslev et al. 1994, D'Eredtia 2001) and in most centres patients now have their NPI calculated after surgery and this is key in the decision making process regarding adjuvant therapy.

1.2.3. Diagnosis and prediction of axillary metastases

With accurate diagnosis of axillary disease being so important in the staging of breast cancer and prediction of prognosis, reliable identification of metastases is vital. Unfortunately clinical examination is very unreliable with approximately one third of clinically 'node negative' patients having metastatic disease histologically and a similar proportion of clinically 'node positive' having no disease histologically (Sacks et al. 1993). Other modalities shown to be inaccurate in staging the axilla include computerised tomography (Isaacs et al. 1993), positron emission tomography (Nieweg et al. 1993) and ultrasonography (Tate et al. 1989). More recently ultrasound has been used to guide FNA or core biopsy of abnormal looking axillary nodes pre operatively. In one study of 39 lymph node positive breast cancer patients, ultrasound guided core was found to be 90% sensitive in the diagnosis of metastatic disease (Topal et al 2005). In another recent study 92.8% of axillary nodes felt to be suspicious on ultrasound were found to harbour metastases (Nori et al 2007). Studies looking at ultrasound-guided FNA have reported much poorer sensitivities at around 55% (Kuenen-Boumeester et al 2003, Lemos S et al 2005). If an ultrasound guided FNA or core biopsy identifies metastatic disease preoperatively then these patients can be excluded from more selective axillary surgery such as sentinel node biopsy.

Many studies have looked at possible predictive factors for axillary metastases. In one such study, tumour size and the presence of lymphovascular invasion were shown to predict axillary involvement on multivariate analysis (Chadha et al. 1994). The incidence of axillary disease in the literature varies from 0% (Mincey et al. 2001) to 12.2% (McGee et al. 1996) for tumours of less than 5mm and from 11.3% (Mincey et al. 2001) to 23.2% (McGee et al. 1996) for tumours of 6 to 10mm. Conversely, a large retrospective study of over 1000 patients with T1 tumours (less than 10mm) showed that tumour size was not a significant predictor of axillary node involvement (Anan et al. 2000). Another study suggested that the size of the invasive component was a better predictor than total tumour size for small cancers (Seidman et al. 1995) and so conflicting evidence may reflect different sizing techniques. There may therefore be a role for selective axillary surgery both for staging purposes as well as local control of disease in patients with small cancers and given that patients are now presenting with smaller symptomatic cancers and screen-detected cancers, a large number of patients could potentially avoid traditional axillary treatment.

1.3. Management of the Axilla

A number of studies have looked at the incidence of axillary disease when the axilla is left untreated. In one such study of over one hundred patients, tumours of less than 10mm had an axillary disease rate of only 10% at 10 years compared with 33% for tumours larger than 21mm (Baxter et al. 1996). The NSABP–B04 trial randomised patients to axillary surgery or no axillary surgery and found an overall axillary recurrence rate of 21% in the untreated group (Fisher et al. 1985). This trial did not however demonstrate a survival advantage in the axillary clearance group suggesting therefore that axillary dissection was a diagnostic rather than a therapeutic procedure. In the NSABP-B04 trial no adjuvant treatment was given and it may have had insufficient numbers to exclude a small survival advantage. A meta-analysis (Orr, 1999) of almost 3000 patients spanning over 4 decades from 6 randomised controlled trials showed an overall survival advantage of 5.4% for axillary clearance. This meta-analysis however had a small

proportion of T1 tumours and essentially no patients had adjuvant therapy and so the risk reduction seen may be diminished by chemotherapy. Axillary recurrence is often devastating when it occurs as it may take some time to become clinically apparent by which time distant metastases are often present. The disease within the axilla itself may produce physical symptoms such as pain and lymphoedema and there are also significant psychological implications.

A recent survey, completed by 371 breast surgeons, of the surgical management of the axilla in the UK was published recently (Gaston et al. 2004). 40% of the surgeons used both axillary dissection and sampling; 28% used axillary dissection alone; 17% used axillary dissection, sampling and sentinel node biopsy; 10% used axillary dissection and sentinel node biopsy and 5% used sampling alone. Of those performing axillary dissection, 49% performed a level III clearance; 42% level II and 9% level I. Of the surgeons performing sentinel node biopsy, 52% were using the technique within a trial setting only and 36% outwith trials only.

1.3.1. Axillary dissection

Axillary dissection or clearance remains the most common means of managing the axilla, in terms of providing staging information and providing local control, in breast cancer patients. A complete (level III) axillary clearance includes the nodes from levels I to III. In the UK level III and level II (levels I and II) axillary clearances are performed in approximately equal numbers (49% versus 42%) with very few surgeons clearing only level I nodes (9%) (Gaston et al. 2004). The decision to perform a level II or III clearance presumably comes down to the surgeons individual preference in most cases and in some cases will be dictated by the presence of abnormal nodes at operation. The surgical boundaries of a level II clearance are the axillary vein superiorly, latissimus dorsi laterally and the medial border of pectoralis minor medially. In a level III clearance nodes medial to pectoralis minor are also excised.

1.3.2. Axillary sampling

Axillary clearance is associated with significant side effects which will be discussed below. Different techniques have therefore been developed in an attempt to accurately stage the axilla without carrying out such an extensive lymphadenectomy. Axillary node sampling was initially introduced as a means of defining patients in whom postoperative radical radiotherapy, including axillary irradiation, could be avoided after simple mastectomy. Reports using axillary sampling as an alternative to clearance started to appear in the literature in the early 1980s. Randomised trials comparing 3 or 4 node sample with axillary clearance in Cardiff and Edinburgh showed no significant difference in the node positivity between the 2 groups and equal survival rates (Chan et al. 1993, Steele et al 1985). A large study advocating the use of axillary sampling carried out a retrospective review of 499 axillary dissection specimens and showed that assessment of 3 to 6 of the largest and firmest lymph nodes in the specimen lead to detection of 93-98% of the node positive patients (Cserni, 1999). Murray et al reviewed 26 patients who had developed axillary recurrences after a 'negative' 4 node axillary sample and found that with serial sectioning and immunohistochemistry, 8% had micrometastases. This was however not statistically different from a control group who had not developed recurrences. This suggested that the recurrences were not due to missed axillary metastases in the sample group.

Some studies however suggest increased axillary recurrence rates (Graverson et al. 1988), and poorer overall survival (Axelsson et al. 1992) when fewer nodes were excised. One study of 960 consecutive patients (Mathieson et al. 1990) showed that the probability of finding at least one positive node increased continuously for up to 10 nodes removed and then levelled off after 10 nodes had been excised at 64% overall node positivity. The authors therefore suggested that at least 10 lymph nodes should be excised to minimise understaging the axilla. This idea has been corroborated by a more recent study in which over 31 000 breast cancer operations were reviewed and in this the node positivity rate was found to increase the more nodes were retrieved. Harvesting over 20 nodes compared to between 10 and 14 nodes increased the node positive rate from 14.2% to 25.9% for tumours up to 5mm in diameter (Axelsson et al 2007).

1.3.3. Axillary radiotherapy

Axillary radiotherapy offers an alternative to surgery in terms of providing locoregional control, however it has no role in staging. In the 1970s there was a trend for simple mastectomy (without axillary dissection) with postoperative radical radiotherapy including axillary irradiation. Currently radiotherapy is most often used to treat the axilla in combination with axillary sampling where the sample reveals positive nodes. In an Edinburgh study patients were randomised to 4 node axillary sampling with subsequent axillary radiotherapy if nodes were involved or a level II axillary clearance with no difference in axillary recurrences between the 2 groups (Forrest et al. 1995). A more extensive randomised study from the same institution has shown similar results as well as reduced morbidity in the sample +/- radiotherapy group (Chetty, 2001).

1.3.4. Sentinel lymph node biopsy

Excision of the first draining 'sentinel' node, which will be discussed in detail below, has been used increasingly to stage the axilla over the past 10 years. It involves lymphatic mapping and therefore selects the lymph node or nodes which are, anatomically, most likely to harbour metastases.

1.4. Morbidity of axillary treatment

The most important cause of morbidity after axillary surgery or radiotherapy is lymphoedema of the corresponding arm. Lymphoedema is usually measured subjectively and therefore often goes unreported. In addition it can take time to develop with the mean time from surgery to onset of lymphoedema being 14 months in one study (Werner et al. 1991). The incidence is similar for both surgery and radiotherapy to the axilla but with surgery the incidence increases the more extensive a dissection is performed (MacKaren et al. 1992). In most studies, surgery and radiotherapy combined produce the highest rates of lymphoedema (Aitken et al. 1989). An extremely rare complication of

lymphoedema is the development of lymphangiosarcoma, Stewart-Treves syndrome (Stewart, Treves. 1948).

Up to a third of patients experience chronic pain after a level III clearance (Ivens et al. 1992). Brachial plexopathy occurs in 5% of patients at between 10 months and 4 years post radiotherapy and results in constant pain and weakness of the arm (Spittle, 1995).

Paraesthesia of the shoulder and upper arm resulting from division of the intercostobrachial nerve occurs in 70-80% undergoing a level II axillary clearance (Lin et al. 1993). Preservation of the nerve results in chronic pain in about a third of patients.

Seromas occur in up to 50% of patients undergoing axillary dissection despite the use of suction drains (Tadych et al. 1987). Postoperative haematomas, wound infections and delayed wound healing are also sources of morbidity.

1.5. Sentinel lymph node biopsy

During the 1950s and 60s various lymphatic mapping studies were reported, including injection of blue dye into the testicle with identification of blue lymphatic channels in the groin (Busch et al. 1963). Several years later breast lymphatics and a blue draining axillary node were imaged using blue dye (Kell et al. 1970). In 1977 the urologist Ramon Cabanas injected blue dye into a squamous carcinoma of the penis and then observed a blue lymph node in the groin (Cabanas, 1977). He described this node as the 'sentinel node', arguing that this was the first node to drain the cancer and that if this node was disease-free then lymphadenectomy was unnecessary. This concept revived Halsted's concept of sequential lymphatic drainage. Around the same time a group from UCLA working on melanoma proposed that lymphatic drainage varied between patients and therefore an intraoperative technique was required to identify each individual's drainage pattern. In this study, initially presented to the WHO in 1989 (Morton et al.

1989) and then published in 1992 (Morton et al. 1992), they carried out lymphatic mapping by injecting blue dye intra-dermally around the primary melanoma in 223 consecutive patients.

Sentinel node biopsy, where lymphatics are traced by some means to one or a few draining sentinel nodes, developed during the early 1990s. Most early reports used the technique in melanoma patients and then increasingly studies emerged using it in the management of breast cancer.

1.5.1. False negative rate

For sentinel node biopsy to be a useful clinical tool it must accurately predict the presence of lymph node metastases. Patients in whom the sentinel node is negative but other draining nodes in the lymphadenectomy specimen are positive are termed 'false negatives'. In breast cancer patients, the false negative rate can only be calculated when sentinel node biopsy is performed in conjunction with an axillary clearance. It is calculated as a percentage of the total number of positive patients (true positives plus false negatives). The false negative rate therefore determines the number of patients who would be incorrectly staged using sentinel node biopsy. Such patients would potentially miss out on adjuvant therapy and therefore have a poorer outcome.

1.5.2. Identification techniques

In Morton's study of melanoma patients (Morton et al. 1992), lymphatic mapping was carried out using isosulphan dye injected around the primary tumour and then blue stained lymphatic channels were traced to the blue sentinel node. They identified at least one blue node in 82% of cases and identified a clear learning curve for the procedure where more experienced surgeons obtained better detection rates. The following year studies were published using injected technetium labelled colloid and a gamma probe to identify radioactive sentinel nodes both in melanoma (Alex et al. 1993) and breast cancer (Krag et al. 1993) patients. A combination of blue dye and radiolabelled colloid was then

used both in melanoma (Albertini et al. 1996) and breast cancer (Hill, 1999) with improved detection rates. Blue dye and radioactive colloid do not always identify the same node(s) and so the two methods complement each other. The American College of Surgeons Oncology Group trial ACOSOG Z0010 (White et al. 2004) which is a phase III trial not yet reported, has shown incidentally that in over 5000 women who underwent sentinel node biopsy using different detection techniques, identification of the sentinel node was similar for dye alone, colloid alone or the combined method. Most centres performing sentinel node biopsy now use the combined approach to improve detection rates and also reduce false negative rates. This obviously depends on having access to nuclear medicine and it is probably more important to have performed a standard technique many times than to always use dye and colloid together. Any node which is blue, or with blue lymphatic channels leading up to it, or radioactive ('hot') or blue and hot is identified as a sentinel node. A 'hot' node has been defined by most studies as a node with a count rate of greater than or equal to 10 times the background radioactivity rate (Cox et al 1998, Bass et al 1999, Sato et al 2002). In cases where more than one hot sentinel node are retrieved, the hottest node is not always the pathologically positive node as shown by one study where in 27.5% of 40 cases examined this was the case. However when the hottest sentinel node retrieved was used as a reference point and 10% of this activity used as the cut off for identifying a node as a sentinel node, the technique was 97.5% sensitive in predicting overall axillary node status (Bourgeois et al 2003).

Two types of blue dye have been used in sentinel node biopsy, isosulphan blue and patent blue, and these are biochemically similar. They bind to albumin and then are selectively taken up by lymphatics and excreted into bile (90%) and urine (10%). There is minimal diffusion into the surrounding soft tissues unlike other dyes such as methylene blue.

In Europe the radioactive colloid used most in sentinel node biopsy is a nanocolloid labelled with technetium 99 which has a particle size of 80nm. In the USA a sulphur colloid, particle size 200nm, is used. The smaller particles of the colloid used in Europe are more rapidly absorbed and so appear in the draining lymph nodes faster. These colloids will accumulate in lymph nodes regardless of whether they contain metastases

and in fact heavily involved lymph nodes often have reduced tracer uptake due to blockage of afferent lymphatic channels by tumour deposits. The dose injected varies in the literature from 7MBq (Veronesi et al. 1997) to 370MBq (Van der Ent et al. 1993). Radioactive nodes can be identified preoperatively using a gamma camera and the site of hot spots marked on the patients skin, and intraoperatively using a hand held gamma probe. The optimum timing of tracer injection and preoperative imaging is debatable and different reports suggest obtaining static images between 2 hours (Borgstein et al. 1998) and 6 hours (Roumen et al. 1997) post injection. The gamma probe detects gamma photons generating an audible output which is then displayed as a 10 second count to quantify the level of radioactivity. The radioactivity of a sentinel node depends on the size of the node and the number of associated afferent lymphatic channels as well as its position in the drainage order. In one study the positive node was the hottest in only 60% of patients in whom more than one sentinel node was identified (Eubus et al. 1999). The probe can be used either with the skin intact or after incision and usually a combination of both is used to identify any hot nodes. The probe is useful after harvesting of the sentinel node to detect any residual radioactivity in the axilla and therefore any additional sentinel nodes.

There has been much debate as to the best injection technique both for blue dye and radioactive colloid. Initially, subdermal and peritumour injection routes were used. Within the breast there is a higher concentration of lymphatics subdermally and so injection under the skin which overlies the tumour results in more rapid uptake of tracer. However it is argued that injection around the tumour itself is more accurate in determining the individual lymphatic drainage pattern. The precise communication between breast and overlying dermal lymphatics is unknown however one study showed complete concordance between the sentinel node of a breast tumour and the overlying skin in 33 patients by using subdermal blue dye and peritumoural radioactive colloid (Borgstein et al. 1997).

An injection technique which has emerged since the sentinel node biopsies were performed in the current study is the subareolar injection route. The first study to look at

this route was published in 1999 and out of 40 cases the detection rate was 98% and the sensitivity for detection of axillary disease was 100% (Kern 1999). Subsequent studies confirming that this technique produced detection rates and false negative rates equivalent to or better than peritumoural injection emerged a few years later (Peley et al 2004, Chagpar et al 2004). One main advantage of this technique is in the management of patients with multifocal breast cancer. In one study of 40 patients with multifocal disease subareolar radioactive colloid was injected and both the detection rate and sensitivity were 100% (Layeeque et al 2003). Two studies have also been published looking at intraoperative subareolar injection of radioisotope. The mean delay between injection of radioisotope and axillary incision was between 15 and 20 minutes and detection rates greater than 95% were achieved in both (Layeeque et al 2004, Zogakis et al 2005).

1.6. Sentinel Lymph Node Biopsy in Breast Cancer

Since the mid 1990s there have been many studies published looking at sentinel node biopsy in breast cancer and in many centres it has now replaced traditional axillary dissection, although still largely on a trial basis. Uptake of the technique in the UK has been slower and it is not yet the standard of care in most UK centres. The Royal College of Surgeons of England is currently running a training programme for sentinel node biopsy ('New Start'). This involves a theory day at a regional centre, which should be attended by as many members of the multidisciplinary breast care team as possible, and then a training day at the trainee team's hospital where 5 sentinel node biopsies are guided by an experienced sentinel node surgeon. An audit phase is then completed in the local hospital with a further 30 cases performed by the same surgeon during which time a satisfactory detection rate and false negative rate should be achieved.

In many patients more than one sentinel node is identified in the axilla. The average numbers of sentinel nodes harvested per patient in a selection of publications available at the start of this study are detailed in table 1:

no. patients	average no SLN/patient	reference
57	2.2	Albertini '96
160	1.4	Veronesi '96
440	1.9	Cox '98
405	2.6	Krag '98
110	1.8	Morrow '99
458	2.1	Hill '99
132	2.1	Giuliano '00
69	2.0	Fissell '01

Table 1: Average number of sentinel nodes found per patient in published series

1.6.1. Initial studies determining accuracy

The initial studies published of sentinel node biopsy in breast cancer concentrated on determining the detection rate, sensitivity and false negative rate and therefore the overall safety of the technique. In these studies a completion axillary dissection was performed following detection of the sentinel node. Table 1 displays a selection of these studies from the literature. Analysis of the sentinel node varied from standard H&E histology (*) to serial sectioning (¤) and immunohistochemistry (°).

author/year	no. patients	technique	DR	FNR
Giuliano'94*	174	dye	65.5%	11.9%
Albertini'97*	62	dye+colloid	92%	0
Veronesi'97¤°	163	colloid	98%	4.7%
Cox'98¤°	466	dye+colloid	94.4%	0.9%
Krag'98*	443	colloid	91.4%	11.4%
Koller'98*	98	dye	97.9%	5.9%
Frisell'01*	75	dye+colloid	92%	11.1%
Tafra'01¤°	535	dye+colloid	87.1%	12.8%
Smillie'01¤°	106	dye+colloid	83.9%	5.5%

Table 2: Sentinel node biopsy studies where completion axillary dissection was performed

DR: detection rate
FNR: false negative rate

1.6.2. Studies without axillary dissection

There are now a number of studies where patients with a negative sentinel node have not had any further axillary surgery or treatment. No long term follow up data regarding axillary recurrence is yet available but, given that the mean time to axillary recurrence is 18 months, the short and medium term follow up data of these studies look very promising. In table 2 the number of patients detailed in each study refers to the number of sentinel node negative patients who did not have an axillary dissection.

author/year	No. patients	MFU	ARR
Schrenk'01	227	22 months	0
Roumen'01	100	24 months	0
Takei'02	358	21 months	0
Reitsamer'04	200	36 months	0
Torrenga'04	104	57 months	0.96%
Veronesi'05	953	38 months	0.31%
Zavagno'05	479	36 months	0
Haid'06	180	47 months	0.56%
Takei'07	822	34 months	1.4%

Table 3: Sentinel node negative studies where no axillary dissection was performed

MFU: mean follow up
ARR: axillary recurrence rate

There were no incidences of distant metastases other than 3 cases (2.9%) in the study by Torrenga et al.

A few studies have recently been published showing medium-term survival data for patients who have undergone sentinel node biopsy alone. At the end of 2006 Veronesi et al published their updated survival data with a median follow up of 79 months (Veronesi et al 2006). This study of over 500 patients reported 5 year survival rates of 96.4% and 98.4% in patients who had undergone axillary clearance and sentinel node biopsy alone respectively. A recent Japanese study of over 1300 patients randomised to sentinel node biopsy alone versus axillary clearance with a median follow up of 31 months has found that the type of axillary surgery has no impact on disease free survival or overall survival (Takei et al 2006).

1.6.3. Trials

There are now several large multi-centre randomised controlled trials underway to assess both the long term safety of the technique and also the reduction in morbidity over conventional axillary treatment.

Within the UK, the ALMANAC trial (Clarke et al. 2001) began in 2001 with an initial audit phase where individual surgeons have to perform 40 sentinel node biopsies and achieve an overall detection rate of 90% and false negative rate of less than 5% before entering the randomised phase comparing sentinel node biopsy where the node was negative versus axillary clearance or 4 node sample. Primary end points include axillary morbidity and quality of life as well as economic issues. Of the first 13 surgeons to complete phase I, all achieved the necessary detection and false negative rates to proceed to phase II (Clarke et al. 2004). Data from the first 1031 patients randomised to sentinel node biopsy alone or axillary clearance/sample has now been published and this shows overall quality of life and arm functioning scores better in the sentinel node biopsy group. In addition there was no increase in anxiety in the group of patients undergoing sentinel node biopsy and there were fewer drains used, shorter hospital stay and reduced time to return to normal activities in this group (Mansel et al 2006).

In the USA the NSABP-B32 trial (Krag et al. 2004) is a phase III multicentre randomised trial comparing sentinel node biopsy with axillary dissection. 217 surgeons from 73 institutions are currently accruing patients who are randomised to level II axillary dissection or sentinel node biopsy with completion axillary dissection only in patients with an H&E positive sentinel node. The primary aim is to determine whether sentinel node biopsy alone provides a similar survival rate and regional control as axillary dissection but with fewer side effects. The secondary aim is to look at the effect of 'occult' metastases within the sentinel node on survival. In the ACOSOG Z0010 trial (White et al. 2004), which has now closed, patients underwent bone marrow biopsy just prior to sentinel node biopsy. IHC was then performed on the bone marrow and sentinel node to determine prognostic accuracy. The results of this trial are awaited.
Another large randomised multicentre phase III trial comparing sentinel node biopsy to axillary dissection is the Royal Australian College of Surgeons's SNAC trial. By 2004 789 out of the target 1000 patients had been recruited (Gill et al. 2004).

1.7. Pathological analysis of sentinel lymph nodes

1.7.1. Paraffin histology

When an axillary clearance is performed, it is unlikely that all axillary nodes are examined histologically or otherwise. Firstly some nodes may be left behind by the surgeon and secondly some small nodes may be missed by the pathologist when the specimen is cleared. Nodes that are retrieved usually undergo a limited examination as analysis of multiple sections by histology and immunohistochemistry (IHC) is not practical. Sentinel node biopsy presents the pathologist with just one or a few nodes rather than 20 or more and so a more detailed analysis of this node is practical.

Analysis of lymph nodes at multiple levels by haematoxylin and eosin (H&E) increases the detection of metastases (Ludwig Group 1990, Pickren 1961, Fisher et al. 1978, Wilkinson et al. 1982). IHC also increases the diagnostic yield and upstages nodes that

are negative by conventional histology (Hainsworth et al. 1993, Byrne et al. 1987, Wells et al. 1984). These techniques allow the detection of smaller metastatic deposits. Micrometastases, defined as deposits smaller than 2mm, and isolated tumour cells can be identified using more sensitive techniques. The clinical significance of such metastases will be discussed below.

At present the optimum pathological handling of sentinel nodes has not been established and there is currently wide variation in practice between different centres. A recently published study (Cserni et al. 2004) in which questionnaires were completed by nearly 400 European pathology units suggested that 70% used IHC routinely if initial H&E histology was negative and 60% performed intra-operative analysis of the sentinel node. Only 4% of units were using molecular staging. Another recent study performed extensive analysis of 173 sentinel nodes, examining each node at 10 levels by H&E and IHC and found that with the addition of 2 additional H&E levels and 1 IHC level (where the initial H&E at one level was negative) correctly identified the sentinel node status in 97.9% of patients (Yared et al. 2002).

Many of the published series of sentinel node biopsy in breast cancer have used serial sectioning and IHC where initial H&E histology was negative (see table 2). The studies detailed in table 4 are examples of those that have looked specifically at the ability of serial sectioning and IHC to upstage sentinel nodes. The upstaged rates given are the result of serial sectioning and IHC combined and are expressed as a proportion of the total number of patients.

Author/year	no. patients	%upstaged
Liu '00	38	19%
Freneaux'02	103	33.9%
Motomura'02	152	13%
Pargaonkar'03	84	7.8%
Klevesath'05	216	4%

Table 4: Use of serial sectioning and IHC to upstage sentinel nodes in breast cancer

1.7.2. Intra-operative analysis

Intra-operative analysis of the sentinel node allows a completion axillary clearance to be performed at the time of initial surgery if the sentinel node is found to be positive. This therefore spares a significant number of patients a second procedure. The reliability of intra-operative analysis depends on the tumour load as well as the size of the node itself. Clearly the more sections are examined, the higher the tumour pick up rate will be. However the examination must be performed fast enough for it to be acted upon in theatre and so a balance must be struck between the number of sections examined and the time and cost involved.

1.7.2.1. Frozen section

A single frozen section can usually be prepared and examined within 30 minutes. Table 5 displays a selection of studies that have examined the accuracy of frozen section analysis of sentinel nodes in breast cancer. There was one false positive in the study by Tanis et al giving a specificity of 99% but in the other 4 studies the technique was 100% specific.

author/year	no. patients	sensitivity
Tanis'01	265	74%
Mitchell'05	874	60%
Al-Shibli'05	70	79%
Brogi'05	133	59%
Grabau'05	272	73%

Table 5: Accuracy of frozen section analysis of sentinel nodes in breast cancer

1.7.2.2. Imprint cytology

Imprint cytology is a faster technique than frozen section but very few cells are examined. In a meta-analysis of 31 studies of imprint cytology of sentinel lymph nodes the overall sensitivity was 63% and specificity 99% (Tew et al. 2005).

1.8. Reverse transcription polymerase chain reaction (RT PCR) and breast cancer

RT PCR is a very sensitive technique which detects and amplifies mRNA. It can detect an isolated tumour cell among one million normal cells. RT PCR has been used to detect different breast and other cancer markers in blood, bone marrow and lymph nodes in an attempt to identify occult metastases. Many breast cancer markers have been studied however tumours display heterogeneous gene expression and so a single mRNA marker may be inadequate for the reliable detection of metastases. In one study which used RT PCR to identify 5 potential breast cancer markers in healthy donor blood, normal lymph nodes and sentinel nodes from breast cancer patients, variable expression was found in the blood and normal nodes (Bostick et al. 1998). There was no single marker which was consistently expressed by histologically positive nodes however combining the results of all 5 markers, 90% of the histologically positive nodes displayed expression of at least one marker. Another study looking at expression of multiple markers by sentinel nodes

found that none of the markers individually had a sensitivity of more than 77.8% but using the results of expression of all 5 markers, the sensitivity was 95.6% (Manzotti et al. 2001). A 2 marker protocol has been suggested by one group who found that the combination of any 2 of the markers they studied gave optimum results and the addition of a third marker to the panel did not improve metastastic detection rates (Fabisiewicz et al 2004). The false negative rate of RT PCR may at least in part be explained by sampling error because, by the nature of the process of RT PCR, the part of the node examined by this technique is not examined histologically and vice versa.

The first report to show that over expression of breast cancer associated genes in patients with histologically node negative breast cancer was correlated with traditional prognostic indicators was published recently (Gillanders et al. 2004). This study used RT PCR to detect 7 different breast cancer markers in 489 patients and found that 33% of histologically negative lymph nodes were RT PCR positive and that RT PCR positivity was significantly associated with prognostic factors such as grade.

The RT PCR results obtained in this study were qualitative however since the laboratory work was carried out real time quantitative PCR has emerged as a technique for examining sentinel nodes. In one recent study this was found to be a more sensitive technique in the diagnosis of metastatic disease than qualitative PCR (Dell 'Orto et al 2006).

1.8.1. Putative breast cancer markers

In the present study the following three breast cancer markers have been selected: cytokeratin 19 (CK 19), mucin 1 (MUC 1) and carcinoembryonic antigen (CEA). The evidence for each of these markers will be discussed in detail below but in general the literature suggests that at least 90% of breast cancers express CK 19 and 100% express MUC 1. The reported expression of CEA is more variable with most studies suggesting that approximately 70% of cancers are CEA positive. The specificity of the three markers is even less easy to establish and expression of all three has been reported in

control blood samples and lymph nodes. Other studies however report negative expression in controls.

	CK 19	MUC 1	CEA
sensitivity	>90%	100%	approx 70%
specificity	?	?	?

Table 6: Sensitivity and specificity of CK 19, MUC 1 and CEA as breast cancer markers

1.8.1.1. Cytokeratin 19

Cytokeratin 19 belongs to a family of intermediate filament proteins expressed by epithelial cells (Moll et al. 1982). Malignant cells generally retain the intermediate filaments of their progenitor cell types and so cytokeratins are used to characterise neoplastic cells of epithelial origin.

Many studies have used immunohistochemistry to verify cytokeratin expression by breast tissue. Bartek used mononclonal antibodies to 2 different epitopes of CK 19 and found that while in benign breast lesions there was heterogeneous expression with up to 50% of the cells remaining unstained, there was homogeneous positive staining in all invasive breast cancers (Bartek et al. 1985). A later study looking at the expression of different cytokeratins using a panel of monoclonal antibodies in benign and malignant breast lesions found that cytokeratin 19 was expressed by all lesions. The expression of some other cytokeratins was more variable (Heatley et al. 1995).

Immunohistochemistry has also been employed to identify CK 19 expression in the axillary nodes, bone marrow or peripheral blood of breast cancer patients. In one study of 358 axillary lymph nodes from breast cancer patients, 5.9% were found to express CK

19 and this compared to 4.5% found to harbour metastases by H&E histology (Inokuchi et al. 2003).

More recently RT PCR has been used in breast cancer patients to determine CK 19 expression. In one study CK 19 expression was found in all of 23 primary breast cancers examined (Noguchi et al, 1996). The expression level of CK 19 mRNA was however found to be variable, although always present to some extent, in a quantitative RT PCR assay looking at 52 primary tumours (Schroder et al. 2003). The sensitivity of CK 19 expression, as determined by RT PCR has not been corroborated by all studies such as that by Papadimitriou which found that 10% of invasive cancers did not express CK 19 (Papadimitriou et al. 1993). Low expression rates have also been found in vitro by the breast cancer cell line MCF 7 (Eltahir et al. 1998).

Inokuchi found that 13.1% of 358 axillary lymph nodes displayed CK 19 expression compared to an H&E positivity rate of 4.5% (Inokuchi et al. 2003). Zhang used RT PCR to analyse axillary drainage fluid and found that CK 19 expression was an independent predictor of locoregional recurrence (Zhang et al. 2006). Many papers have reported the CK 19 expression of peripheral blood in breast cancer patients such as that by Xenidis which reported CK 19 expression in 21.6% of 167 node negative breast cancer patients and in addition these patients had a significantly increased risk of early relapse or disease related death (Xenedis et al. 2006). The detection of CK 19 in peripheral blood has also been used to monitor response to neoadjuvant chemotherapy and in one study of 53 patients there was a significant reduction in CK 19 expression after chemotherapy (Manhani et al. 2001).

Variable specificity rates for CK 19 expression have been found in the literature. In one study where peripheral blood from 30 healthy controls and from 15 patients having benign breast biopsies were subjected to RT PCR, no expression of CK 19 was found (Kahn et al. 2000). Datta found similar results with only one of 39 controls displaying CK 19 expression in peripheral blood and the single positive was a patient with chronic myelogenous leukaemia (Datta et al. 1994). However two studies have each shown CK

19 expression in around one fifth of control blood samples (Wong et al. 2001, Krismann et al. 1995).

Few studies have included normal lymph nodes in their evaluation of CK 19 expression by RT PCR and they have looked at only a few nodes each. The absence of CK 19 expression by normal lymph nodes was found by some studies (Traweek et al. 1993, Noguchi et al. 1996) but in another a few normal nodes did express CK 19 (Bostick et al, 1998).

1.8.1.2. Mucin 1

Mucin 1 (MUC 1) is a transmembrane high molecular weight glycoprotein which is important for cell adhesion. It is also known as CA 15.3 and PEM (polymorphic epithelial mucin) and is uniformly and highly expressed by primary breast tumours. The overexpression seen in breast cancer cells results in reduced cell to cell and cell to extracellular matrix adhesion and therefore allows cell detachment. Overexpression of MUC 1 has also been found to induce T cell apoptosis (Gimmi et al. 1996).

Since the 1980s many authors have used monoclonal antibodies to detect MUC 1 in the serum of breast cancer patients. In a large retrospective multicentre study of 500 patients over a 7 year period, the preoperative serum MUC 1 level was found to correlate with stage, tumour size, lymph node status and the presence of metastases (O'Hanlon et al. 1995). MUC 1 level was not however found to be an independent prognostic factor in this study. Elevated levels of MUC 1 were found in the peripheral blood in 68.4% of 98 patients with advanced or recurrent breast cancer (Iwase et al. 1995). Another study of 252 patients found a significant correlation between elevated serum MUC 1 and locoregional recurrence and time to development of metastatic disease, with a mean lead time of 9.9 months between elevation of MUC 1 and diagnosis of metastases (Coveney et al. 1995). Pectasides found that monitoring serum MUC 1 was 68.2% sensitive in detecting disease progression in a study of 209 patients (Pectasides et al. 1996). A study with longer follow up data of 5 years or more in 103 patients found that MUC 1 levels

were 61.1% sensitive and 91.2% specific for detecting cancer recurrence (Lumanchi et al. 1999).

Serum MUC 1 levels have been used less successfully to monitor response to breast cancer therapy. In one study of 99 breast cancer patients undergoing neoadjuvant chemotherapy the MUC 1 serum level did not change after chemotherapy even when there was a good clinical response however an elevated pre-treatment level was associated with an increased recurrence rate (Bottini et al. 1997).

Breast cancer mucin has a novel carbohydrate epitope and is therefore antigenically distinct from normal breast tissue mucin. The heavily sialylated side chain of breast cancer MUC 1 has greater immunogenicity and the monoclonal antibodies that have been developed have been considered in the development of anticancer vaccines as well as being used to detect serum MUC 1 as described above. A live recombinant vaccinia virus coexpressing human MUC 1 and the human cytokine IL 2 was used in 9 breast cancer patients in whom there were increased numbers of T memory cells and no adverse reactions (Scholl et al. 2000).

Identification of MUC 1 mRNA by RT PCR has been carried out in primary tumours, axillary nodes, peripheral blood and bone marrow. In one study 10 out of 10 histologically positive axillary nodes also expressed MUC 1 and 6% of 53 histologically negative nodes were upstaged (Noguchi et al. 1996). The presence of MUC 1 in peripheral blood was found in 24% of patients with operable breast cancer compared to 45% of patients with advanced disease in another study of 122 patients (deCremoux et al. 2000).

Some studies have suggested that MUC 1 is a specific breast cancer marker which is rarely expressed by non epithelial tissues (Zotter et al. 1988, Noguchi et al. 1994). It has however been detected in peripheral blood and lymph nodes from healthy controls (Bostick et al. 1998).

1.8.1.3. Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a tumour antigen associated with poorly differentiated tumour cells. Initial publications suggesting that serum CEA could be used as a breast cancer marker appeared in the mid 1970s. Many studies then assessed serial CEA evaluation as a means of monitoring the disease. Some found that elevated CEA was correlated with subsequent progression and that the rise in levels were seen several months before relapse was clinically apparent (Lokich et al. 1978, Myers et al. 1978). Similar results were found in a more recent study of 135 patients in which a rise in serum CEA was found on average 7 months before relapse was clinically apparent (Jezersek et al. 1996).

As well as being used to monitor disease progression, CEA has been found to be an independent prognostic factor in breast cancer by some studies (Mansour et al. 1983, Ebeling et al. 1999). The study by Ebeling of 550 patients without metastatic disease at diagnosis found that the preoperative CEA level was an independent prognostic factor for overall survival on multivariate analysis. In a recent study of 103 patients with locally advanced breast cancer undergoing neoadjuvant chemotherapy, serum CEA was found to be an independent predictor of outcome (Martinez-Trufero et al. 2005). Serum CEA has also been shown to be significantly correlated with the size of the primary tumour and also axillary node status (Lumanchi et al. 2000).

CEA is not however universally expressed by breast cancer cells. Overall expression rates by primary tumours of 65% and 75% were found in two different studies (Halter et al. 1984, Marchetti et al. 2001). Not surprisingly other studies have failed to verify CEA as an accurate predictor of progression such as one large study of 664 breast cancer patients where serial CEA had a diagnostic accuracy of 83% for relapse and a positive predictive value of only 27% (Sutterlin et al. 1999). The sensitivity of serum CEA was only 27% for detection of visceral metastases and 46% for detection of bony metastases in another study of over 1000 patients (Given et al. 2000). Elevation of CEA occurred in

only 38% of 549 patients with recurrent disease in a study with at least 5 years follow up (Guadagni et al. 2001).

1.8.2. RT PCR analysis of sentinel lymph nodes

RT PCR has been used to evaluate sentinel lymph nodes from breast cancer patients in several studies and a few examples are listed in table 7 below. There are also now some reports of using real time RT PCR to analyse sentinel lymph nodes (Schroder et al. 2003). Despite relatively few studies there are now commercially available RT PCR kits which are being targeted at the analysis of sentinel lymph nodes.

author/year	no. patients	markers(s)	% upstaged
Peley'01	68	CK20	20.6%
Branagan'02	50	Mammoglobin	8%
Sakaguchi'03	108	CK19/EGP2	16%
Weigelt'04	70 nodes	CK19/p1B/EGP2/SBEM	10%

Table 7: Studies using RT PCR to analyse sentinel lymph nodes in breast cancer

1.9. Axillary micrometastases

A micrometastasis is usually defined as a metastatic deposit with a maximum diameter of less than 2mm. Very small metastases are now also referred to as isolated tumour cells (ITC).

1.9.1. Impact of axillary micrometastases on survival

Initial studies published in the 1960s and 70s suggested that 'occult' axillary node metastases or micrometastases detected by serial sectioning or IHC did not influence survival (Pickren et al. 1961, Fisher et al. 1978, Huvos et al. 1971, Attiyeh et al. 1977). An important study which found that micrometastases did reduce survival was that published by the Ludwig group in 1990. It found that micrometastases were present in 9% of 921 patients using serial sectioning and IHC and found that these patients had a 16% reduction in disease free survival. Other studies which have also found a reduction in survival associated with axillary micrometastases are summarised in table 8.

author/year	no. patients	incidence MM	DFS reduction
Trojani'87	162	14%	22%
Sedmark'89	45	11%	11%
Ludwig Gp'90	921	9%	16%
Chen'91	80	29%	16%
deMascarel'92	218	23%	10%
Hainsworth'93	343	12%	15%

Table 8: Studies showing reduced survival associated with axillary micrometastases

MM: micrometastases
DFS: disease free survival

Some studies have looked at whether sentinel node micrometastases can predict the presence of metastatic disease elsewhere in the axilla. Of these, some have shown that they do predict other disease (Viale et al. 2001, Schrenk et al 2005) and others have shown no correlation (Fournier et al. 2004, Rutledge et al. 2005). Those that show no correlation between sentinel node micrometastases and non sentinel node disease

involved small numbers as shown in table 9. The number of patients given for each study refers to the number of patients found to have a sentinel node containing micrometastatic disease only.

author/year	no.patients	% positive NSLN
Viale'01	109	21.8%
Schrenk'05	122	18%
Fournier'04	16	6.2%
Rutledge'05	29	3%

Table 9: Prediction of non sentinel node metastases by sentinel node micrometastases.

NSLN: non sentinel lymph node

A few studies have now been published looking at the effect of sentinel node micrometastases on survival. Chagpar found that in 15 patients micrometastases of the sentinel node identified by IHC there was no reduction in incidence of distant metastases, overall survival or disease free survival (Chapgar et al. 2005). Sakaguchi performed RT PCR on pooled sentinel and non sentinel nodes from 108 patients and found that 16% expressed either CK19 or epithelial glycoprotein 2 and were therefore upstaged (Sakaguchi et al. 2003). The 4 year survival for patients that had metastases identified by RT PCR only was 100%, compared with 74% for patients with axillary metastases diagnosed by conventional histology.

1.9.2. Summary

The evidence that axillary lymph node micrometastases influence survival is not clear cut. Further research by long term follow up studies is required to answer this question once and for all. Micrometastases have been identified by different authors using different techniques – IHC, serial sectioning and now at the molecular level by RT PCR. The ideal target for RT PCR analysis is the sentinel node(s), given that this is the node most likely to harbour metastases and that only one or few nodes need to be processed. There is, however, limited data on RT PCR evaluation of sentinel nodes from breast cancer patients. The aim of this thesis was to use the technique to identify 3 different breast cancer markers in sentinel lymph nodes from breast cancer patients. The number of nodes 'upstaged' in relation to H&E histology were compared when different amounts of tissue from sentinel nodes were processed.

Chapter 2
Materials and Methods

2.1. Ethical approval

A study proposal was submitted to the research and development office of North Glasgow University Hospitals NHS Trust. Ethical and financial approval were granted on 4th November 2000 (project number 00SG011).

2.2. Patient selection and follow up

The selection criteria for sentinel lymph node biopsy were as follows. Patients were those attending the one stop breast clinic at Glasgow Royal Infirmary with a solitary, biopsy-proven invasive breast cancer. Clinically node positive patients and patients undergoing immediate breast reconstruction were excluded. All diagnoses were confirmed by core biopsy, following FNA and mammography +/- breast ultrasound. When the patients returned for the results of their core biopsy, the operative options were discussed with the patients along with an explanation of the study. Patients were given an information leaflet (appendix 1) about the study to take home. Patients not undergoing reconstruction were then admitted for surgery within the following 2 weeks. All patients who participated in the study signed a patient consent form (appendix 2) in addition to the usual consent form for surgery following admission to hospital.

From 1996 to 1999, 54 patients underwent sentinel node biopsy at Glasgow Royal Infirmary, the preliminary results of which have been previously published (Flett et al. 1998). Sixty eight sentinel nodes were harvested from these patients (average 1.26 sentinel nodes per patient). These nodes were bisected and half the node stored at -80°C. These half nodes were used for RT PCR analysis. These patients are referred to as 'group 1' below.

During a 16 month period beginning January 2001, 50 patients underwent sentinel node biopsy at Glasgow Royal Infirmary and in these patients RT PCR was performed on a

4μm section cut from each node leaving the remainder of the node for conventional paraffin histology. These patients are referred to as 'group 2' below.

2.3. Sentinel lymph node biopsy

The sentinel node biopsies carried out in group 2 were all performed using a combination of radioactivity and blue dye as previously described (Albertini et al. 1997, Cox et al. 1998, Frisell et al. 2001, Tafra et al. 2001, Smillie et al. 2001). All of these cases were performed by the author together with a consultant breast surgeon or subspecialty breast trainee. The sentinel node biopsies carried out in group 1 (prior to the start of the present study) were performed using blue dye in all cases and radioactive colloid in 56% of the cases.

The detection rates achieved and in particular the high number of negative preoperative scintigrams compare poorly with many published series. The technique used to harvest the sentinel nodes prospectively was based on the literature available at the time. Since then different injection techniques have emerged. These cases represented an early phase of the technique for the investigating institution and for the surgeons involved. The technique has evolved in Glasgow Royal Infirmary and the results of the technique itself today compare very differently with these early results. As the aim of this thesis was to evaluate RT PCR in the analysis of sentinel nodes and comparison of these results with conventional histology, all of the cases were included.

2.3.1. Lymphatic mapping

On the day prior to surgery the patients who had radioactive colloid used (all group 2 and 56% of group 1) were injected with technetium 99-labelled colloid (nanocoll). A maximum dose of 40MBq was used, contained in a volume of 1-2ml. This was injected at 4 peritumoural sites (at 12, 3, 6 and 9 o'clock) and a small volume (<0.5ml) was injected sub-dermally over the tumour site. The injection technique was carried out in

the same way for all patients and was always by the investigator for the group 2 patients. Two hours later a scan was performed using a gamma camera to identify any axillary hot spots. If any were seen, they were marked on the patients skin at the corresponding point. Intra-operatively, a gamma-probe (C-Trak™) was used to identify radioactivity. Any sentinel nodes removed were checked for radioactivity with the probe and if any signal was obtained a 10 second count was determined using the probe and recorded.

2.3.2. Blue dye

After induction of general anaesthesia, patent blue dye was injected in all cases from groups 1 and 2. 1ml dye was diluted up to a total volume of 5ml in 0.9% saline. Four separate injections of 1ml each were then administered peritumourally at 12, 3, 6 and 9 o'clock and the final 1ml was injected subdermally over the tumour. The area was then massaged briefly and the patient prepped. A minimum of 10 minutes elapsed between injection and surgery.

2.3.3. Operative technique

Patients were prepped and draped in a standard fashion after injection of blue dye. The patient's arm on the operating side was positioned at 90°. The gamma probe was scanned over the patient's axilla before any incision was made. A curved incision was then made in the lower axilla through skin and subcutaneous fascia. Dissecting scissors were then used to explore the tissue. The probe was used at regular intervals to check for any hot spots and blue stained lymphatic channels searched for in the cases where radioactive colloid had been used. When either of these were identified they were traced to the sentinel node. After the sentinel node had been excised the probe was used again where appropriate to check for any residual activity and again blue stained lymphatics searched for. In general, the dissection was carried from the lower axilla upwards. Details of each operative case were recorded including start and finish times for the procedure overall and the sentinel node biopsy itself.

All patients in both groups proceeded to a level II axillary clearance after the sentinel node had been harvested.

2.4. Pathology

Any identified sentinel nodes were taken immediately to the pathology department to avoid unnecessary degradation of RNA.

2.4.1. Frozen section

The sentinel node was incised and a portion used to prepare 2 frozen sections. Wherever possible, the hilum of the node was identified and the node was then bivalved along its long axis. Sections were then cut at a thickness of 4μm and one was then stained with H&E and given to the duty consultant pathologist for real time reporting. The remaining section was put onto dry ice and transported immediately to a -80°C freezer where it remained until the RNA was extracted.

2.4.2. Paraffin histology

All sentinel nodes were bisected and each half examined at 3 histological levels. All other axillary nodes were bisected and each half examined at 1 level. Any node bigger than 6mm in diameter was cut into 3mm slices and each slice examined at 3 levels for sentinel nodes and 1 level for non sentinel nodes. This protocol has been ratified by the most recent pathology guidelines from the NHS breast screening programme (2005).

2.5. RNA extraction

All plastic ware was pre-treated with di-ethylpyrocarbonate (DEPC) and autoclaved. DEPC is a potent RNAse inhibitor. At all times latex gloves were used when handling

tubes and tissue to prevent RNAse contamination. Gloves were changed frequently and tubes kept closed whenever possible.

RNA extraction techniques were worked up using breast tissue prior to using sentinel node tissue. The sentinel nodes were taken from theatre to the pathology department as soon as they had been harvested. The frozen sections were prepared within 5 minutes of the tissue arriving in the department. The section cut for RNA extraction was put onto dry ice immediately and then stored at -80° C until use.

2.5.1. DEPC treatment of microfuge tubes/distilled water.

Distilled water was DEPC treated by adding 1.5ml DEPC to 1.5L water and mixing for 24 hours. Microfuge tubes were treated by immersing in 0.1% DEPC treated water overnight. The tubes were then dried and autoclaved to eliminate residual DEPC.

2.5.2. RNA Isolation using the TRIzol (GibcoBRL Life Technologies) method

Small pieces (0.5–1g) of previously frozen breast tissue were crushed and immediately placed in TRIzol (1ml TRIzol per 100mg tissue) at room temperature. The tissue sample in TRIzol was then homogenised using a PowerGen 125 tissue homogeniser (Fisher Scientific) starting at 5000 RPM and gradually going up to approximately 20 000 RPM over a period of 30-60 seconds at room temperature. This resulted in a homogeneous solution with very few visible tissue pieces. The sample was then incubated at room temperature for 5-10 minutes after homogenisation. The TRIzol/tissue homogenate was then transferred to a 50ml centrifuge tube and centrifuged at 10 000 RPM for 5-10 minutes at 4 °C. The upper fat layer was removed using a pasteur pipette. 0.2ml chloroform per 1ml TRIzol reagent used was added and the tube shaken vigorously for 15-30 seconds by hand and incubated at room temperature for 5 minutes. The tube was then spun at 10 000 RPM for 15 minutes at 4 °C. The upper aqueous phase, containing the total RNA, was then removed and placed in a new 50ml centrifuge tube. The RNA was then precipitated by adding 0.5ml isopropylalcohol per 1ml TRIzol reagent used.

The sample was incubated at room temperature for 10 minutes, spun at 10 000 RPM for 10 minutes at 4°C and then left at -20°C overnight. The next day the tubes were defrosted and spun at 12 000 RPM for 20 minutes at 4°C. The supernatant was then decanted and the pellet washed once using 1ml 75% ethanol per 1ml TRIzol. This was then spun at 7 500 RPM for 5 minutes at 4°C. The supernatant was decanted and the pellet air dried at room temperature for 10-20 minutes. The pellet was then resuspended in 50 µl DEPC-treated water and incubated at room temperature for 10 minutes and then stored at 4°C.

When this technique was used to extract RNA from frozen sections, the sections were scraped from the glass slide using a scalpel and added to tubes containing 1ml TRIzol. The remaining steps followed as above.

2.5.3. RNA extraction using the Qiagen RNeasy kit

When RNA was extracted from breast tissue or sentinel nodes rather than frozen sections, a maximum of 30mg tissue was used as suggested by the manufacturers. This tissue had been snap frozen following operation and stored at -80°C to prevent RNA degradation. The tissue was transferred from the freezer to a cold mortar and pestle and liquid nitrogen added. The tissue was then ground to a powder and transferred to a 1.5ml microfuge tube containing 600µl buffer RLT. Before use, β mercaptoethanol (β ME) was added to buffer RLT (10µl β ME per 1ml buffer RLT). The sample was further homogenised using a white needle attached to a 5ml syringe – the sample was aspirated using the needle multiple times. The lysate was then added to a QIA shredder column in a 2ml collection tube and centrifuged for 2 minutes at 12 000 RPM. The lysate was then transferred to a 1.5ml microfuge tube and centrifuged for 3 minutes at 12 000 RPM. The supernatant was then transferred to another microfuge tube and an equal volume of 70% ethanol added and mixed by pipetting. The full volume was then added to a spin column sitting in a 2ml collection tube and centrifuged for 15 seconds at 12 000 RPM. The flow through was discarded and 700µl buffer RW1 added to the column. This was again centrifuged for 15 seconds at 12 000 RPM and the flow through discarded. The column

was then transferred to a new 2ml collection tube and 500µl buffer RPE added to the column. Buffer RPE was supplied as a concentrate and 4 volumes 100% ethanol was added prior to use. The column was then centrifuged for 15 seconds at 12 000 RPM and the flow through discarded. A further 500µl buffer RPE was added to the column and centrifuged for 2 minutes at 12 000 RPM. The column was then placed in a new 2ml collection tube and centrifuged for 1 minute at 12 000 RPM. The column was transferred to another new collection tube and 30µl RNAse-free water pipetted onto the column. This was centrifuged for 1 minute at 12 000 RPM to elute the RNA. RNA samples were then immediately stored at -20°C.

When RNA extractions were performed from frozen sections, the tissue was scraped off the glass slide using a scalpel blade into a 1.5ml microfuge tube containing 350µl buffer RLT (with β ME added as before). The tube was then vortexed briefly and the lysate pipetted onto the QIA shredder. The following steps were as described before.

2.6. Reverse transcription reaction.

Extracted RNA samples were reverse transcribed using the Omniscript ™ Reverse Transcriptase kit. Template RNA solutions were thawed on ice. Primers were not included in the kit and that used was oligo-dT primer (invitrogen). The primer solution along with the other reagents – 10x buffer RT, dNTP mix, omniscript reverse transcriptase and RNAse-free water were thawed at room temperature and then stored on ice. Each solution was mixed by vortexing and centrifuged briefly to collect residual liquid from the sides of the tubes. The following master mix was then prepared: 2µl 10x buffer RT, 2µl dNTP mix, 2µl oligo dT, 1µl reverse transcriptase and 8µl RNAse-free water. This mix was then vortexed for 5 seconds and centrifuged briefly. 5µl RNA solution was then added to the master mix, vortexed for 5 seconds and centrifuged briefly. The mixture was then incubated at 37°C for 1 hour.

2.7. Polymerase chain reaction

The CAG repeat and β actin PCRs were worked up and well established in the laboratory. These PCRs, which confirm the presence of house keeping genes, were used at various points in the study to confirm the presence of cDNA after RNA extraction and reverse transcription. The PCR reactions for the breast cancer markers CK 19, MUC 1 and CEA had to be optimised and this process is detailed in chapter 6. In addition to these PCRs, 2 other reactions were tried at the start of this study. These were to determine the expression of cytokeratin 20 (CK 20) and mammaglobin however after failing to get these reactions working these markers were abandoned. Different master mixes, primer sequences and PCR conditions were tried for each marker initially using control DNA (extracted from blood) and then using DNA from breast tissue until a correctly sized band was reliably produced. The reagents, primer sequences and conditions tried were taken from previously published studies. In all cases the following reagents were added to a microtube before placing on the PCR block - 2μl DNA, 2μl primer mix, 10μl master mix and 6μl water. The total volume in each tube was therefore 20μl. The master mixes, primer sequences and conditions ultimately used together with the product sizes are detailed in tables 10 - 13 below.

CAG repeat/β actin/CK 19	**MUC 1**	**CEA**
200 μl buffer IV *	200 μl 10 x buffer °	200 μl 10 x buffer °
200 μl dNTPs	200 μl dNTPs	200 μl dNTPs
10 μl Taq polymerase ¤	10 μl Taq polymerase ¤	10 μl Taq polymerase ¤
120 μl $MgCl_2$		120 μl $MgCl_2$
470 μl H_2O	590 μl H_2O	470 μl H_2O

Table 10: PCR master mixes (total volume for each = 1ml).

*Advanced Biotechnologies (containing 15mM $MgCl_2$)
° Qiagen (containing 1.5mM $MgCl_2$)
¤ Advanced Biotechnologies

PCR	primer sequences	reference
CAG repeat	5'-TCC GCG AAG TGA TCC AGA AC-3'; 5'-CTT GGGG AGA ACC ATC CTC A -3'	Mir et al. 2002
β actin	5'-TAT CCA GGC TGT GCT ATC CCT GTA C-3'; 5'-CTT GAT GAG GTA GTC AGT CAG GTC C-3'	designed in house
CK 19	5'-AGG TGG ATT CCG CTC CGG GCA-3'; 5'-ATC TTC CTG TCC CTC GAG CA-3'	Noguchi et al. 1996
MUC 1	5'-CGT CGT GGA CAT TGA TGG TAC C-3'; 5'-GGT ACC TCC TCT CAC CTC CTC CAA-3'	Bostick et al. 1998
CEA	5'-TCT GGA ACT TCT CCT GGT CTC TCT CAG CTG G-3'; 5'-GGG CCA CTG TCG GCA TCA TGA TTG -3'	Ooka et al. 2000

Table 11: PCR primer sequences

β actin/MUC 1/CEA	CAG repeat	CK 19
step 1 95°C x 5 minutes step 2 95°C x 1 minute step 3 50°C x 1 minute step 4 72°C x 1 minute (steps 2-4 x 35 cycles) step 5 72°C x 10 minutes	step 1 95°C x 1 minute step 2 95°C x 1 minute step 3 50°C x 1 minute step 4 72°C x 1 minute (steps 2-4 x 25 cycles) step 5 72°C x 10 minutes	step 1 95°C x 5 minutes step 2 95°C x 1 minute step 3 60°C x 1 minute step 4 72°C x 2 minutes (steps 2-4 x 25 cycles) step 5 72°C for 10 minutes

Table 12: PCR conditions

PCR product	Size
CAG repeat	163 bp
β actin	169 bp
CK 19	160 bp
MUC 1	288 bp
CEA	160 bp

Table 13: PCR product sizes

The presence of the three markers was determined in the sentinel nodes from groups 1 and 2. The primary tumours were not examined. The aim of this study was to compare the presence of the markers with each other and with conventional histology of the sentinel nodes. If the technique were to prove sensitive in the detection of metastases then RT PCR using one or a combination of these markers may have a staging role in clinical practice. In the clinical setting, the marker expression by the sentinel nodes and not the primary tumour would be determined by RT PCR as the aim of the technique is to detect axillary metastases. Accepting that CEA expression by breast cancers in particular is variable, the combination of the results of two or all three markers should identify metastatic disease if the technique is a reliable tool.

2.8. Agarose gel electrophoresis

A stock solution of 50x TAE (tris acetic acid EDTA) was prepared as follows:

242g tris acetic acid was added to 500ml distilled water. 100ml 0.5M Na2EDTA (pH 8) and 57ml glacial acetic acid were then added and the total volume adjusted to 1l. To make 1x TAE this solution was diluted 50 times in distilled water.

Loading buffer was prepared as follows:

30ml 30% glycerol was added to 70ml water. 25mg 0.25% xylene cyanol and 25mg 0.025% bromophenol blue were then added. Before loading samples to an agarose gel, 5µl of loading buffer was added to each sample.

A 2% agarose gel was prepared as follows:

5g agarose was added to 250ml 1x TAE and microwaved for 2½ minutes. This was left to cool and then 10µl ethidium bromide solution (10mg/ml) was added and mixed gently. The solution was then poured into a gel rig and left to set. Samples (with loading buffer added) were then placed in the wells. In one lane 100bp DNA ladder was run (1µl ladder + 9µl loading buffer) in order to size any bands seen. The gel was then run for approximately 90 minutes at 50-75 volts. The gel was then visualised under UV fluorescence and photographed.

Chapter 3
Patient Results

3.1. Group 1

There were 54 patients from whom 68 half sentinel nodes had been stored at -80°C. All patients had unilateral cancers and so the number of procedures was equal to the number of patients. All patients were female. Thirty one patients underwent mastectomy and 23 underwent breast conserving surgery. The results for this group are given in detail below (table 14) and then summarised (table 15).

pt no.	age	no. SLN	wts. of ½ SLNs	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI
1	67	1	0.27g	0	0/10	IDC	80mm	3	5.6
2	47	2	0.11g 0.15g	1	0/8	IDC	12mm	3	5.24
3	86	1	0.99g	0	0/8	IDC	35mm	3	4.7
4	48	1	0.27g	0	0/10	IDC	12mm	1	2.24
5	56	1	0.16g	0	0/8	IDC	14mm	2	3.28
6	71	1	0.19g	0	0/15	IDC	30mm	2	3.6
7	65	1	0.18g	0	0/12	IDC	55mm	2	4.1
8	50	1	0.27g	0	0/23	IDC	28mm	2	3.56
9	35	2	0.18g 0.91g	0	0/11	IDC	10mm	1	2.2
10	64	2	0.14g 0.10g	0	0/13	ILC	16mm	2	3.32
11	69	1	0.15g	1	0/15	IDC	29mm	3	5.58
12	53	2	0.07g 1.1g	0	0/15	ILC	13mm	1	2.26
13	73	2	0.3g 0.14g	0	0/16	IDC	35mm	2	3.7
14	49	1	0.92g	0	0/9	IDC	16mm	2	3.32
15	79	3	0.37g 041g 1.2g	3	8/22	IDC	36mm	3	6.72
16	73	1	0.42g	0	0/8	ILC	25mm	2	3.5
17	76	1	0.33g	0	0/18	tubular	20mm	1	2.4
18	43	1	0.15g	0	1/12	IDC	11mm	2	4.22
19	50	1	0.26g	0	0/9	IDC	6mm	1	2.12
20	73	1	0.58g	0	0/17	IDC	22mm	2	3.44
21	53	1	0.05g	0	0/17	IDC	22mm	3	4.44
22	68	1	0.48g	0	0/8	IDC	21mm	2	3.42
23	64	1	0.62g	1	0/14	IDC	40mm	3	5.8
24	35	1	0.1g	0	0/15	IDC	12mm	3	4.24
25	47	2	0.22g 0.38g	0	0/26	IDC	15mm	1	2.3
26	73	1	0.09g	0	0/16	IDC	20mm	2	3.4
27	39	1	0.26g	0	1/19	IDC	13mm	3	5.26
28	56	1	0.17g	0	0/6	IDC	18mm	1	2.36
29	64	1	0.18g	0	0/14	IDC	15mm	2	3.3
30	72	1	0.3g	0	0/10	IDC	35mm	2	3.7
31	63	1	0.43g	0	0/15	IDC	18mm	3	4.36
32	46	1	0.6g	0	0/13	tubular	10mm	1	2.2

pt no.	age	no. SLN	wts. of ½ SLNs	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI
33	33	1	0.95g	0	0/15	IDC	16mm	3	4.32
34	73	2	0.14g 0.09g	0	0/13	IDC	9mm	1	2.18
35	59	1	0.28g	0	0/14	IDC	12mm	3	4.14
36	50	1	0.05g	0	8/18	ILC	15mm	2	5.3
37	54	2	0.07g 0.15g	0	0/16	IDC	23mm	1	2.46
38	56	1	0.08g	0	0/17	IDC	24mm	1	2.48
39	48	2	0.10g 0.25g	0	0/30	IDC	15mm	3	4.3
40	78	1	0.06g	0	0/11	ILC	50mm	2	4
41	56	1	0.97g	0	0/14	IDC	6mm	2	3.12
42	82	1	0.29g	0	0/14	IDC	25mm	3	4.5
43	69	1	0.06g	0	0/10	IDC	22mm	2	3.44
44	53	2	0.5g 0.03g	1	0/10	IDC	30mm	3	5.6
45	45	2	0.11g 0.21g	0	0/14	IDC	10mm	3	4.2
46	55	2	0.3g 0.35g	0	0/5	IDC	14mm	2	3.28
47	39	1	0.13g	0	0/19	IDC	10mm	2	3.2
48	71	1	0.5g	0	0/12	mucoid	14mm	1	2.28
49	45	1	0.07g	0	0/16	IDC	14mm	2	3.28
50	80	1	0.32g	1	1/11	IDC	31mm	2	4.62
51	56	1	0.07g	0	0/9	IDC	22mm	2	3.44
52	65	1	0.51g	0	2/12	IDC	25mm	3	5.5
53	63	1	0.04g	0	0/11	IDC	13mm	1	2.26
54	69	1	0.27g	0	0/18	IDC	19mm	2	3.38

Table 14: Group 1 data

number sentinel lymph nodes(no. SLN); number positive sentinel lymph nodes (no. +ve SLN); weights of half sentinel lymph nodes (wts. ½ SLN); number positive non sentinel axillary nodes (no. other +ve LN); tumour type (T type); tumour size (T size); grade; Nottingham Prognostic Index (NPI)

Group 1	54 patients
age range	33-86 (average 58.8)
% SLN only positive	7.4% (4/54)
% NSLN only positive	3.7% (2/54)
% SLN + NSLN positive	7.4% (4/54)
FNR	40% (4/10)
% node positivity	10/54 (18.5%)
tumour size	6-80mm (average 21.4mm)
% Grade I / II / III	24.1% / 44.4% / 31.5%
% invasive ductal carcinoma	85.2
NPI	2.12-6.72 (average 3.72)

Table 15: Group 1 - summary of patient details

3.2. Group 2

During a 16 month period sentinel node biopsy was attempted in 64 patients. One patient had bilateral cancers making a total of 65 procedures. A sentinel node was detected in 50 patients giving a detection rate of 77%. The bilateral case had a sentinel node detected on one side only and so the total number of procedures included in the study was also 50.

Of the 15 procedures where no sentinel node was detected, the age range was 52 to 72 (average 62). Tumour size ranged from 10 to 50 mm (average 20.4mm). In 9 cases (60%) the tumour was medially located. The pre-operative scan was negative in 12 out of 15 procedures (80%). Of the 3 positive scans, the hot spot was identified in the internal mammary chain in 2 cases and the axilla in one case. The overall axillary node status was positive in 10 out of 15 cases (66.6%) and in 3 of these cases the axilla was found to be macroscopically positive at operation. In addition 3 of the cases where no sentinel node was found were significantly overweight. The dose of radioactivity given

ranged from 23 to 40 MBq with an average dose of 37.7 MBq per patient. The range in time delay between injection of radioactive colloid and the operation start time was 18 to 25 hours (average delay 21.4 hours). The delay between injection of blue dye and the operation start time ranged from 10 to 15 minutes with an average delay of 11.2 minutes.

Of the 50 patients in whom a sentinel node was found, one was male. Thirty cancers were left sided and 20 right sided. Twenty nine patients (58%) underwent breast conserving surgery while 21 (42%) underwent mastectomy. In 46 cases (92%) the histological type was invasive ductal carcinoma as shown below and of the remaining 4 cases, 2 were invasive lobular carcinomas and 2 were special type tubular/mucoid carcinomas. The results from the 50 patients where a sentinel node was harvested are given in detail below (table 16) and then summarised (table 17). The 15 procedures from which no sentinel node was harvested have not been included in this table and will not be discussed any further.

pt no.	age	no. SLN	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI
1	58	1	0	0/12	IDC	9mm	1	2.18
2	53	2	2	6/14	IDC	19mm	3	6.38
3	57	1	0	0/10	IDC	9mm	1	2.18
4	71	1	0	0/10	IDC	20mm	3	4.4
5	78	1	0	0/13	tubular	20mm	1	2.4
6	62	1	1	1/17	IDC	16mm	2	4.32
7	55	1	0	0/10	IDC	9mm	2	2.18
8	70	1	0	0/21	IDC	34mm	3	4.68
9	36	1	0	0/16	IDC	15mm	2	3.3
10	52	1	1	0/12	IDC	10mm	1	2.2
11	55	3	0	0/14	IDC	23mm	3	4.48
12	37	1	1	1/17	IDC	15mm	3	5.3
13	56	2	2	0/11	IDC	15mm	2	4.3
14	52	1	1	20/23	IDC	30mm	3	6.6
15	61	1	0	0/13	IDC	40mm	1	2.8
16	72	1	0	0/14	IDC	15mm	2	3.3
17	75	1	1	11/16	IDC	20mm	1	4.4
18	52	1	1	0/12	IDC	30mm	3	4.6
19	58	2	1	0/13	IDC	20mm	1	3.4
20	81	1	1	0/23	IDC	35mm	2	4.7
21	72	2	0	0/17	IDC	27mm	2	3.54
22	54	1	0	0/6	IDC	15mm	2	3.3
23	36	1	1	6/21	IDC	32mm	3	6.64
24	82	2	0	0/6	IDC	17mm	2	3.34
25	84	1	1	1/13	IDC	47mm	3	5.94
26	50	1	0	1/15	IDC	15mm	2	4.3
27	55	1	0	1/20	IDC	13mm	3	5.26
28	78	2	1	0/5	IDC	15mm	2	4.3
29	74	1	1	2/14	ILC	33mm	2	4.6
30	57	1	0	0/14	IDC	18mm	3	4.36
31	38	1	0	0/13	IDC	12mm	3	4.24
32	79	1	1	11/23	IDC	54mm	3	7.08
33	51	1	0	0/12	tubular	25mm	1	2.5
34	73	1	0	0/13	IDC	45mm	3	4.9
35	61	1	0	9/13	IDC	26mm	3	6.52
36	60	1	1	0/16	IDC	25mm	2	4.5
37	66	1	1	0/16	IDC	20mm	2	4.4
38	79	1	0	0/14	IDC	20mm	3	4.4
39	59	1	1	1/10	IDC	16mm	2	4.32
40	72	1	0	2/14	IDC	12mm	3	5.24

pt no.	age	no. SLN	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI
41	70	1	1	1/14	IDC	33mm	3	5.66
42	67	1	0	0/13	IDC	38mm	3	4.72
43	70	2	1	0/12	IDC	30mm	3	5.6
44	65	1	1	0/8	IDC	13mm	3	5.26
45	56	1	0	0/11	IDC	22mm	2	3.44
46	53	2	1	10/22	ILC	32mm	2	5.64
47	72	1	0	0/14	IDC	22mm	3	4.44
48	65	1	0	0/20	IDC	23mm	3	4.46
49	57	1	0	0/16	IDC	15mm	2	3.3
50	64	1	1	0/15	IDC	42mm	3	5.8

Table 16: Group 2 data

age; number sentinel lymph nodes (no. SLN); number positive sentinel lymph nodes (no. +ve SLN); number positive non sentinel axillary lymph nodes (no. other +ve LN); tumour type (T type); tumour size (T size); grade; Nottingham Prognostic Index (NPI)

Group 2	**50 patients**
age range	36-84 (average 62.3)
% SLN only positive	22% (11/50)
% NSLN only positive	8% (4/50)
% SLN + NSLN positive	24% (12/50)
FNR	14.8% (4/27)
Overall % node positivity	54% (27/50)
tumour size	9-54mm (average 31.5mm)
% grade I / II / III	16% / 36% / 48%
% invasive ductal carcinoma	92% (46/50)
% oestrogen receptor positive	82% (41/50)
NPI	2.18-7.08 (average 4.5)

Table 17: Group 2 - summary of patient details

3.3. Summary and comparison of groups 1 and 2

The total number of patients included in the study was therefore 104. From these patients 128 sentinel nodes were harvested. In total 52 (50%) of patients underwent mastectomy and 52 underwent breast conserving surgery. Table 18 compares the results for the 2 groups:

	Group 1 (54 patients)	**Group 2** (50 patients)
age range	33-86 (average 58.8)	36-84 (average 62.3)
% SLN positive	11.1% (6/54)	46% (23/50)
% overall node positivity	18.5%	54%
FNR	40% (4/10)	14.8% (4/27)
tumour size	6-80mm (av. 21.4mm)	9-54mm (av.31.5mm)
% grade I / II / III	24.1% / 44.4% / 31.5%	16% / 36% / 48%
% invasive ductal Ca	85.2%	92%
NPI	2.12-6.72 (av. 3.72)	2.18-7.08 (av. 4.5)

Table 18: Comparison of groups 1 and 2

Although irrelevant to the purposes of this study, the patients in group 2 which were accrued prospectively during the study represent a prognostically poorer group compared to group 1. The group 2 tumours were generally larger, of higher grade and had a higher rate of axillary disease as detected by paraffin histology. This is reflected in the overall higher average NPI of 4.5 compared with 3.72 in group 1.

One would expect around one third of all breast cancers to be node positive and so group 1 represents a group with fewer than predicted node positive cancers and group 2 has more node positive cases. The patients from group 2 represented all the clinically node

negative patients who presented during the study period agreeing to participate and in whom a sentinel node was identified. The patients were not selected in any other way.

Chapter 4
Sentinel lymph node biopsy technique results

The total number of sentinel nodes harvested from the 54 patients in group 1 was 68 (mean number of sentinel nodes per patient 1.26) and from the 50 patients in group 2 was 60 (mean 1.2).

Sentinel nodes from the 50 patients in group 2 were identified using the combination of radioactive colloid and blue dye. The time taken to identify a sentinel node ranged from 3 minutes to 30 minutes (median 16.5 minutes); the average time taken was 16.5 minutes. The time for the entire procedure ranged from 45 minutes to 115 minutes (median 80 minutes) with an average operation time of 75.6 minutes.

4.1. Lymphatic mapping

The dose of nanocoll injected ranged from 19 to 40 MBq (median 29.5 MBq); the average injected dose 36.6 MBq. The delay between injection of radioactive colloid and surgery ranged from 5.5 to 25 hours (median 15.25 hours); the average delay was 20.9 hours.

In 34 of the 50 patients comprising group 2 (68%) the pre-operative scan did not show any hot spots. In 15 patients one or more hot spots were seen in the axilla on the scan. In the remaining one patient 2 internal mammary hot spots were visualised. Of the 15 procedures also carried out in group 2 where no sentinel node was identified, 12 had a negative scan. Overall therefore the pre operative scan was negative in 70.8% (46/65 procedures).

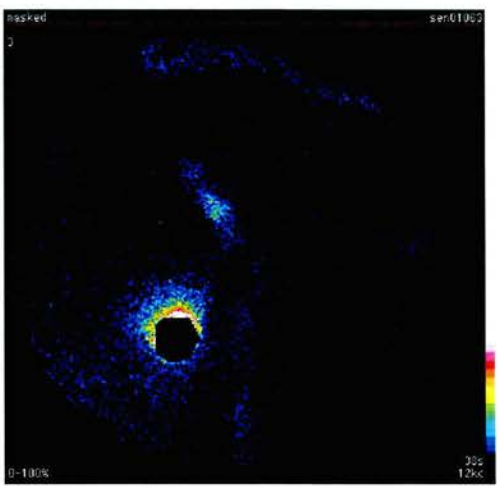


Figure 1: Scan showing hot spot in left axilla. The injection site in the breast has been masked.

Thirty sentinel nodes from 26 patients were radioactive. The majority - 83.3% (25 nodes) - of these nodes were also blue with the remaining 16.6% (5 nodes) being 'hot only'. The 10 second count from the hot nodes ranged from 8 to 439; the average 10 second count was 144.5.

Of the 15 patients in whom an axillary hot spot had been seen on the scan and the patient marked, 14 went on to have at least one hot sentinel node harvested. In the other patient, one blue only sentinel node was found and the failure to identify a hot node from this patient suggests that at least one other sentinel node was missed ie not detected. There were 13 hot sentinel nodes from 11 patients who had had negative pre-operative scans.

4.2. Blue dye

The time delay between injection of blue dye and the start of the procedure ranged from 10 minutes to 15 minutes with an average delay of 10 minutes 23 seconds. Fifty five sentinel nodes from 48 patients were blue. Thirty of these nodes were blue only and, as above, 25 nodes were hot and blue. Figure 2 demonstrates a case where blue stained lymphatics were identified and traced to a blue sentinel node which is shown in figure 3 after excision.

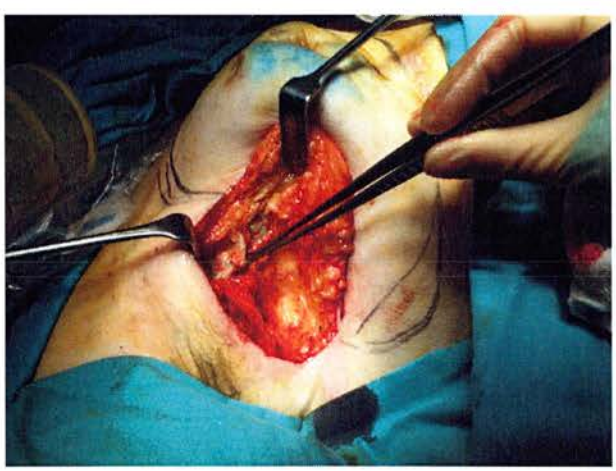


Figure 2: Intraoperative photo showing dissection of tissues following incision of skin in lower axilla to find blue stained lymphatic channels

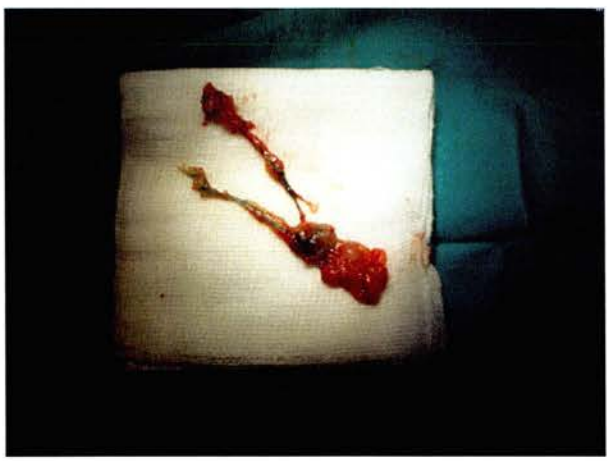


Figure 3: Photograph showing blue sentinel node and 2 blue stained afferent lymphatic channels

4.3. Summary

For the group 2 patients, more sentinel nodes were identified by blue dye than by radioactive colloid. The pre-operative scan was positive in only 32% of these patients.

Chapter 5
Sentinel lymph node histology results

5.1. Intra operative frozen section

Intra-operative frozen section was performed on 43 of the 68 sentinel nodes from group 1 and on all 60 sentinel nodes from group 2. For the group 2 cases, this was reported intra operatively and the results telephoned to the operating theatre with a time range of 19 to 30 minutes – the average time for reporting of results was 25 minutes. The frozen section was reported as positive in 19 sentinel nodes and negative in 84 sentinel nodes. Seven sentinel nodes from 7 patients had a negatively reported frozen section but subsequent H&E histology of the node was positive. This represents a false negative rate for frozen section of 21.2% (7/33) on a node basis. There were no false positive frozen section results.

5.2. Post operative (paraffin) histology

In total, 95 sentinel lymph nodes from 75 patients were H&E negative and 33 sentinel lymph nodes from 29 patients were H&E positive. The overall axillary node status was positive in 37 out of 104 patients (35.6%). Of the node positive patients, 15 had a positive sentinel node and no other positive nodes; 14 had a positive sentinel node and other positive axillary nodes and the remaining 8 patients had a negative sentinel node but other non-sentinel axillary nodes that were involved. This represents a false negative rate, based on H&E histology, of 21.6% (8/37 patients).

The total number of axillary nodes obtained, including the sentinel nodes, ranged from 3 to 24 per patient with an average of 14.75 nodes per patient.

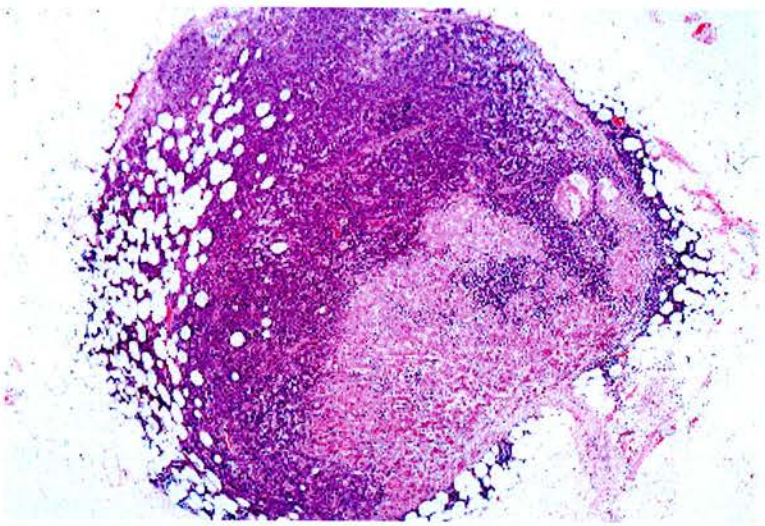


Figure 4: H&E positive sentinel lymph node

5.3. Summary

Table 19 summarises the above results:

% patients with H&E positive SLN	27.9% (29/104)
overall axillary node positive	35.6% (37/104)
FNR	21.6% (8/37)

Table 19: H&E histology results for groups 1 and 2 combined

SLN: sentinel lymph node
FNR: false negative rate

Chapter 6
Development of RT PCR technique

6.1. RNA extraction and reverse transcription

6.1.1. RNA extraction using TRIzol method

RNA extraction was initially attempted using the TRIzol method as described. The initial attempt was made using 0.6g frozen normal breast tissue. A pellet was obtained after the overnight incubation and the resuspended sample used in a CAG repeat PCR. The subsequent RNA gel which was run with and without 2M NaOH added to the sample wells was blank. The technique was therefore repeated using larger tissue samples – 0.8g and then 1g frozen breast tissue but again no bands were obtained after CAG repeat PCR.

Despite not achieving success in extraction of RNA from breast tissue, the technique was tried using frozen sections. Initially 4 x 10μm frozen sections of breast tissue were scraped off into the TRIzol and the technique then continued as before but again no bands were seen on running the resuspended pellet samples on a gel in wells with and without NaOH.

The next step was to attempt a reverse transcription reaction using the superscript ™ system on the samples obtained from the RNA extraction from frozen sections. CAG repeat and β actin PCRs were then performed but no bands were seen on subsequent gel electrophoresis.

6.1.2. RNA extraction using Qiagen RNeasy kit

Following the lack of success in extracting RNA either from solid tissue or frozen sections, another method was tried. The Qiagen RNeasy kit was used initially on a 30mg sample of normal breast tissue. A reverse transcription reaction was carried out using the omniscript™ system and a β actin PCR confirmed the presence of cDNA. The successful extraction of RNA and subsequent successful reverse transcription allowed both of these steps to be carried out on the 68 frozen half sentinel nodes (group 1). The weight of each

half frozen node was determined before RNA extraction. The range of these weights was 0.03g to 1.2g (average 0.31g).

RNA extraction was then worked up on frozen sections. Initially 10x 4μm sections were cut fresh from a breast cancer specimen and RNA extraction and reverse transcription steps carried out as before. Correctly sized bands confirmed the presence of cDNA after βactin PCR. The same process was then carried out using 4x 4μm sections and then a single section and again bands were seen on the gel. This allowed the process to be carried out on the prospectively collected frozen sections (group 2) as they were obtained. Again, reverse transcription was always carried out immediately to allow confirmatory CAG repeat and β actin PCRs.

6.2. Polymerase chain reactions

6.2.1. Cytokeratin 19

The CK 19 PCR was the first reaction to be worked up. Initially this was done using control DNA extracted from blood.

The conditions, primer sequences and master mix used have been detailed in the materials and methods chapter but before these were arrived at alternative master mixes containing a different buffer ('buffer II') and different concentrations of Taq polymerase were tried. Annealing temperatures of 50°C and 55°C were also tried.

Using the conditions described previously a single band was reliably produced on a gel however this was found to be approximately 700bp in size, differing from the 460bp size stated in the literature (Noguchi et al. 1996). However when the sequence of the whole gene was examined the length of the product between the primers was found to be 716bp and when the reaction was then tried on samples where RNA was first extracted from

breast tissue and then a reverse transcription step performed, a correctly sized 460bp product was obtained.

6.2.2. Mucin 1

The PCR for MUC 1 was worked up using samples where RNA had been extracted from breast tissue, reverse transcription performed and presence of cDNA confirmed. Before a single correctly sized band was reliably produced different master mixes and PCR conditions were tried. Master mixes containing different buffers – buffer IV and Q buffer were used and annealing temperatures of 55°C and 60°C tried. The conditions and reagents ultimately used were shown above.

6.2.3. Carcinoembryonic antigen

The PCR for CEA took the longest time to work up and looking back this is almost certainly due to the fact that many of the cDNA samples used in the work up of the reaction were probably CEA negative. Many conditions and master mixes were tried before a single band was produced and indeed different primers were also tried. The initial primer set was as follows: 5'-GGG CCA CTG TCG GCA TCA TGA TTG-3' and 5'-TGT AGC TGT TGC AAA TGC TTT AAG GAA GAA GC-3'. The corresponding product size was 131 bp. Master mixes containing buffer IV and Q buffer and annealing temperatures of 51, 53, 55 and 60°C were all tried at various times. Eventually new primers were ordered, comprising the original reverse primer but a new forward primer and using these with the 10xbuffer master mix containing additional $MgCl_2$ and annealing temperature 50°C, a 160 bp product was achieved.

Chapter 7
RT PCR Results

For each of the 3 markers studied, the sensitivity, specificity and false negative rate have been calculated. The term 'false negative' has already been used above to describe histologically negative sentinel nodes in patients with other positive axillary nodes and is being used here in another context. In order to calculate these, the number of true positives, true negatives, false positives and false negatives must be determined. These have been defined as shown in table 20 below.

	H&E +	**H&E -**
RT PCR +	True positive	False positive
RT PCR -	False negative	True negative

Table 20: Definition of true negatives, false negatives, true positives and false positives by RT PCR compared to H&E

7.1. Cytokeratin 19

7.1.1. Group 1

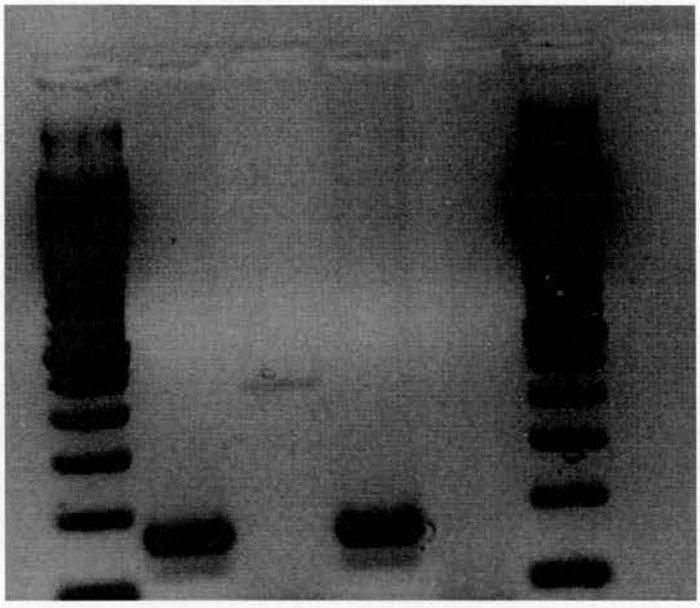
CK 19 expression was found in 25 of the 68 half nodes (36.8%) from group 1. Of the 25 nodes, 16 were from patients that had H&E negative sentinel and non-sentinel nodes, representing an upstaging rate of 23.5%. Seven nodes agreed with positive histology and 2 sentinel nodes were false negatives (1 with another positive sentinel node and the other with another positive non-sentinel node). Of the 43 sentinel nodes that did not express CK 19, there was 1 node that had been H&E positive. This node may have represented a failure of RT PCR and the expression of CK 19 by this node could, in retrospect, have been checked by immunohistochemistry. Of the remaining 42 nodes where negative CK 19 expression did correlate with negative H&E histology, there were 4 false negatives that were not upstaged (1 where another sentinel node had been positive and 1 where another non-sentinel node had been positive).

The sensitivity of CK 19 expression was therefore 90%, specificity 72.4% and false negative rate 10%. The concordance between CK 19 expression and H&E histology was 72% (49/68 nodes).

7.1.2. Group 2

Of the 60 sentinel nodes examined, 15 nodes (25%) were found to express CK 19 by PCR and 45 (75%) were found not to express CK 19. Of the 15 CK 19 positive nodes, all had positive paraffin histology. Of the 45 CK 19 negative nodes, 36 had a histologically negative sentinel node but the remaining 9 nodes had been positive by H&E. Again, CK 19 expression by these nodes could have been further validated by immunohistochemistry. No patients were therefore upstaged by CK 19 PCR and in particular none of the false negative sentinel nodes were upstaged.

This represents a sensitivity of 62.5%, specificity 100% and false negative rate 37.5%. The concordance between the H&E histology of the sentinel node and CK 19 PCR was 85% (51/60).



lane 1 2 3 4 5 6

Figure 5: Gel photo for CK 19 PCR

Lanes 1 and 6 show a 100bp DNA ladder. Lanes 2 and 3 display DNA from one sentinel node where lane 2 shows a 169bp β actin band and lane 3 shows a 460bp CK 19 band. DNA from a different sentinel node has been run in lanes 4 and 5 and lane 4 confirms presence of cDNA (β actin band) but there is no CK 19 band and hence no CK 19 expression.

7.2. Mucin 1

7.2.1. Group 1

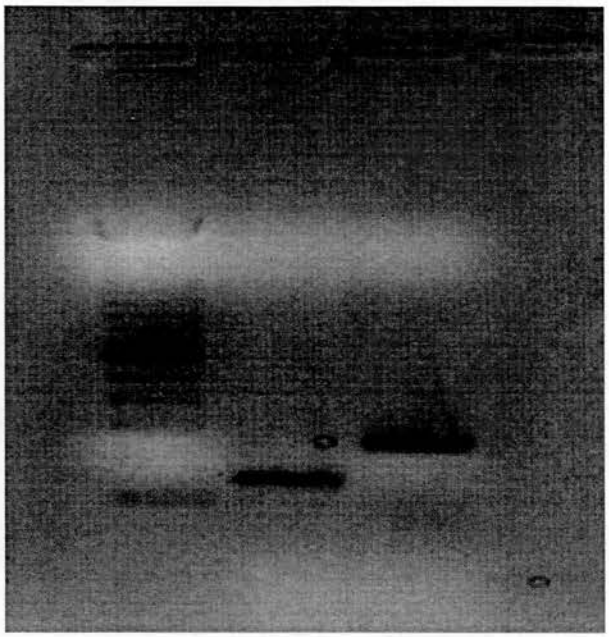
MUC 1 expression was found in 32 out of 68 half nodes (47%). Thirty six nodes (53%) did not express MUC 1. Of the 32 nodes that were MUC 1 positive, 5 had been positive by H&E histology. The remaining 27 sentinel nodes (39.7%) therefore had been upstaged – these included 2 false negative sentinel nodes – 1 with another sentinel node that was H&E positive and 1 with other positive non-sentinel nodes. Of the 36 nodes that were MUC 1 negative, 3 had been H&E positive and 33 H&E negative. There were 4 false negative sentinel nodes included in this group that were not upstaged by MUC 1 PCR.

The sensitivity was therefore 62.5%, specificity 55% and false negative rate 37.5%. Concordance between H&E histology and MUC 1 expression was 55.9% (38/68 nodes).

7.2.2. Group 2

MUC 1 expression was found in 47 out of 60 nodes (78.3%). Thirteen nodes (21.6%) were MUC 1 negative. Of the nodes that expressed MUC 1, 21 nodes were also positive by paraffin histology. Twenty six sentinel nodes (43.3%) were upstaged by MUC 1 PCR. Twenty two of these nodes were H&E negative but 4 were false negatives – 2 with other non-sentinel axillary node(s) involved and 2 with another sentinel node that was H&E positive. Of the 13 nodes that were not found to express MUC 1, 11 were also H&E negative but the other 2 nodes were H&E positive.

The sensitivity was therefore 91.3%, specificity 29.7% and false negative rate 8.7%. The overall concordance between H&E histology and MUC 1 expression was 53.3%.



lane 1 2 3 4

Figure 6: Gel photo for MUC 1 PCR

Lane 1 shows a 100bp DNA ladder. Lanes 2 and 3 display DNA from one sentinel node where lane 2 shows a 169bp β actin band and lane 3 shows a 288bp MUC 1 band.

7.3. Carcinoembryonic Antigen

7.3.1.Group 1

CEA expression was found in 18 out of 68 half nodes (26.5%). Fifty sentinel nodes (73.5%) did not express CEA. Of the 18 CEA positive nodes, only 1 had been H&E positive. The 17 sentinel nodes (25%) that were upstaged by CEA PCR included 1 false negative. Of the 50 CEA negative nodes, the H&E histology had also been negative in 43 cases and this group included 5 false negative nodes – 3 with other non-sentinel node involvement and 2 with another positive sentinel node. There were 7 cases where H&E was positive but no CEA expression detected.

The sensitivity was therefore 12.5%, specificity 71.7% and false negative rate 87.5%. Overall concordance between H&E histology and CEA expression was 64.7% (44/68 nodes).

7.3.2. Group 2

CEA expression was found in 10 out of 60 nodes (16.7%). Fifty nodes (83.3%) did not express CEA. Of the 10 nodes that were CEA positive, 6 were H&E positive but 4 were H&E negative and so 6.7% of all sentinel nodes were upstaged by CEA PCR. None of these upstaged nodes represented any of the false negatives by H&E. Of the 50 nodes that were found not to express CEA, 33 agreed with negative paraffin histology and these included the false negatives - 5 cases where other non-sentinel nodes were positive and 2 cases where another sentinel node was positive. In 17 nodes CEA expression was not found but H&E histology was positive.

The sensitivity was therefore 26.1%, specificity 89.2% and false negative rate 73.9%. The overall concordance between H&E histology and CEA expression was 65%.

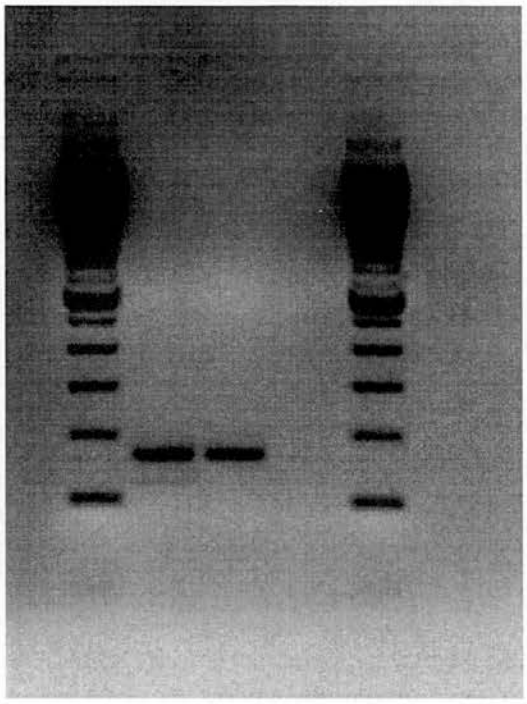


Figure 7: Gel photo for CEA PCR

Lanes 1 and 5 show a 100bp DNA ladder. Lanes 2 and 3 display DNA from one sentinel node where lane 2 shows a 169bp β actin band and lane 3 shows a 166 bp CEA band.

7.4. Summary of RT PCR results

Tables 21 and 22 below display the patient and tumour data from groups 1 and 2 respectively that were previously shown (tables 14 and 16) with the marker expressions of the sentinel lymph nodes as determined by RT PCR added.

pt no.	age	no. SLN	wts. of ½ SLNs	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI	CK 19	MUC 1	CEA
1	67	1	0.27g	0	0/10	IDC	80mm	3	5.6	+	+	-
2	47	2	0.11g 0.15g	1	0/8	IDC	12mm	3	5.24	+ +	+ +	- -
3	86	1	0.99g	0	0/8	IDC	35mm	3	4.7	+	+	-
4	48	1	0.27g	0	0/10	IDC	12mm	1	2.24	-	-	+
5	56	1	0.16g	0	0/8	IDC	14mm	2	3.28	-	-	-
6	71	1	0.19g	0	0/15	IDC	30mm	2	3.6	+	+	-
7	65	1	0.18g	0	0/12	IDC	55mm	2	4.1	-	+	-
8	50	1	0.27g	0	0/23	IDC	28mm	2	3.56	-	+	-
9	35	2	0.18g 0.91g	0	0/11	IDC	10mm	1	2.2	- +	+ -	- -
10	64	2	0.14g 0.10g	0	0/13	ILC	16mm	2	3.32	+ +	+ +	- +
11	69	1	0.15g	1	0/15	IDC	29mm	3	5.58	+	-	-
12	53	2	0.07g 1.1g	0	0/15	ILC	13mm	1	2.26	- -	- -	+ -
13	73	2	0.3g 0.14g	0	0/16	IDC	35mm	2	3.7	- -	- +	+ -
14	49	1	0.92g	0	0/9	IDC	16mm	2	3.32	+	+	-
15	79	3	0.37g 041g 1.2g	3	8/22	IDC	36mm	3	6.72	+ + +	+ + +	+ - -
16	73	1	0.42g	0	0/8	ILC	25mm	2	3.5	-	-	-
17	76	1	0.33g	0	0/18	tubular	20mm	1	2.4	-	-	-
18	43	1	0.15g	0	1/12	IDC	11mm	2	4.22	-	-	+
19	50	1	0.26g	0	0/9	IDC	6mm	1	2.12	-	-	+
20	73	1	0.58g	0	0/17	IDC	22mm	2	3.44	-	-	+
21	53	1	0.05g	0	0/17	IDC	22mm	3	4.44	-	-	+
22	68	1	0.48g	0	0/8	IDC	21mm	2	3.42	+	+	-
23	64	1	0.62g	1	0/14	IDC	40mm	3	5.8	+	+	-
24	35	1	0.1g	0	0/15	IDC	12mm	3	4.24	-	-	+
25	47	2	0.22g 0.38g	0	0/26	IDC	15mm	1	2.3	- -	- +	+ -
26	73	1	0.09g	0	0/16	IDC	20mm	2	3.4	-	+	-

pt no.	age	no. SLN	wts. of ½ SLNs	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI	CK 19	MUC 1	CEA
27	39	1	0.26g	0	1/19	IDC	13mm	3	5.26	-	-	-
28	56	1	0.17g	0	0/6	IDC	18mm	1	2.36	-	-	-
29	64	1	0.18g	0	0/14	IDC	15mm	2	3.3	-	+	+
30	72	1	0.3g	0	0/10	IDC	35mm	2	3.7	+	-	+
31	63	1	0.43g	0	0/15	IDC	18mm	3	4.36	+	+	-
32	46	1	0.6g	0	0/13	tubular	10mm	1	2.2	+	-	+
33	33	1	0.95g	0	0/15	IDC	16mm	3	4.32	+	+	-
34	73	2	0.14g 0.09g	0	0/13	IDC	9mm	1	2.18	- -	+ -	- -
35	59	1	0.28g	0	0/14	IDC	12mm	3	4.14	-	-	-
36	50	1	0.05g	0	8/18	ILC	15mm	2	5.3	+	+	-
37	54	2	0.07g 0.15g	0	0/16	IDC	23mm	1	2.46	- -	+ -	- +
38	56	1	0.08g	0	0/17	IDC	24mm	1	2.48	-	+	+
39	48	2	0.10g 0.25g	0	0/30	IDC	15mm	3	4.3	- -	- -	- -
40	78	1	0.06g	0	0/11	ILC	50mm	2	4	-	+	+
41	56	1	0.97g	0	0/14	IDC	6mm	2	3.12	+	-	-
42	82	1	0.29g	0	0/14	IDC	25mm	3	4.5	-	-	-
43	69	1	0.06g	0	0/10	IDC	22mm	2	3.44	-	-	-
44	53	2	0.5g 0.03g	1	0/10	IDC	30mm	3	5.6	- -	- -	- -
45	45	2	0.11g 0.21g	0	0/14	IDC	10mm	3	4.2	+ -	+ -	+ -
46	55	2	0.3g 0.35g	0	0/5	IDC	14mm	2	3.28	+ +	+ +	- -
47	39	1	0.13g	0	0/19	IDC	10mm	2	3.2	-	-	-
48	71	1	0.5g	0	0/12	mucoid	14mm	1	2.28	-	-	-
49	45	1	0.07g	0	0/16	IDC	14mm	2	3.28	-	-	-
50	80	1	0.32g	1	1/11	IDC	31mm	2	4.62	+	-	-
51	56	1	0.07g	0	0/9	IDC	22mm	2	3.44	-	-	-
52	65	1	0.51g	0	2/12	IDC	25mm	3	5.5	-	-	-
53	63	1	0.04g	0	0/11	IDC	13mm	1	2.26	-	+	-
54	69	1	0.27g	0	0/18	IDC	19mm	2	3.38	-	+	-

Table 21: Group 1 patient and tumour data with sentinel lymph node marker expression (CK19, MUC 1 and CEA) (+ denotes marker expression)

pt no	age	no SLN	no +ve SLN	no. other +ve LN	T type	T size	grade	NPI	CK 19	MUC 1	CEA
1	58	1	0	0/12	IDC	9mm	1	2.18	-	+	-
2	53	2	2	6/14	IDC	19mm	3	6.38	+ +	+ +	- -
3	57	1	0	0/10	IDC	9mm	1	2.18	-	+	-
4	71	1	0	0/10	IDC	20mm	3	4.4	-	+	-
5	78	1	0	0/13	tubular	20mm	1	2.4	-	+	-
6	62	1	1	1/17	IDC	16mm	2	4.32	-	-	-
7	55	1	0	0/10	IDC	9mm	2	2.18	-	+	-
8	70	1	0	0/21	IDC	34mm	3	4.68	-	-	-
9	36	1	0	0/16	IDC	15mm	2	3.3	-	+	-
10	52	1	1	0/12	IDC	10mm	1	2.2	-	-	-
11	55	3	0	0/14	IDC	23mm	3	4.48	- - -	+ + +	- - -
12	37	1	1	1/17	IDC	15mm	3	5.3	-	-	-
13	56	2	2	0/11	IDC	15mm	2	4.3	+ +	+ +	+ -
14	52	1	1	20/23	IDC	30mm	3	6.6	+	+	-
15	61	1	0	0/13	IDC	40mm	1	2.8	-	-	-
16	72	1	0	0/14	IDC	15mm	2	3.3	-	-	-
17	75	1	1	11/16	IDC	20mm	1	4.4	+	+	-
18	52	1	1	0/12	IDC	30mm	3	4.6	-	+	-
19	58	2	1	0/13	IDC	20mm	1	3.4	+ -	+ -	+ -
20	81	1	1	0/23	IDC	35mm	2	4.7	+	+	+
21	72	2	0	0/17	IDC	27mm	2	3.54	- -	+ +	+ +
22	54	1	0	0/6	IDC	15mm	2	3.3	-	+	-
23	36	1	1	6/21	IDC	32mm	3	6.64	+	+	-
24	82	2	0	0/6	IDC	17mm	2	3.34	- -	+ -	- -
25	84	1	1	1/13	IDC	47mm	3	5.94	+	+	-
26	50	1	0	1/15	IDC	15mm	2	4.3	-	+	-
27	55	1	0	1/20	IDC	13mm	3	5.26	-	+	-

pt no.	age	no. SLN	no. +ve SLN	no. other +ve LN	T type	T size	grade	NPI	CK 19	MUC 1	CEA
28	78	2	1	0/5	IDC	15mm	2	4.3	-	-	-
29	74	1	1	2/14	ILC	33mm	2	4.6	-	+	+
30	57	1	0	0/14	IDC	18mm	3	4.36	-	+	+
31	38	1	0	0/13	IDC	12mm	3	4.24	-	+	-
32	79	1	1	11/23	IDC	54mm	3	7.08	+	+	-
33	51	1	0	0/12	tubular	25mm	1	2.5	-	+	+
34	73	1	0	0/13	IDC	45mm	3	4.9	-	-	-
35	61	1	0	9/13	IDC	26mm	3	6.52	-	-	-
36	60	1	1	0/16	IDC	25mm	2	4.5	-	+	-
37	66	1	1	0/16	IDC	20mm	2	4.4	-	+	+
38	79	1	0	0/14	IDC	20mm	3	4.4	-	+	-
39	59	1	1	1/10	IDC	16mm	2	4.32	-	+	-
40	72	1	0	2/14	IDC	12mm	3	5.24	-	-	-
41	70	1	1	1/14	IDC	33mm	3	5.66	+	+	-
42	67	1	0	0/13	IDC	38mm	3	4.72	-	+	-
43	70	2	1	0/12	IDC	30mm	3	5.6	- -	+ +	- -
44	65	1	1	0/8	IDC	13mm	3	5.26	+	+	-
45	56	1	0	0/11	IDC	22mm	2	3.44	-	+	-
46	53	2	1	10/22	ILC	32mm	2	5.64	+ -	+ +	+ -
47	72	1	0	0/14	IDC	22mm	3	4.44	-	+	-
48	65	1	0	0/20	IDC	23mm	3	4.46	-	-	-
49	57	1	0	0/16	IDC	15mm	2	3.3	-	+	-
50	64	1	1	0/15	IDC	42mm	3	5.8	+	+	-

Table 22: Group 2 patient and tumour data with sentinel lymph node marker expression (CK 19, MUC 1 and CEA)

Table 23 below summarises the sensitivities, specificities and false negative rates of marker expression by RT PCR compared with H&E histology for groups 1 and 2.

	sensitivity	specificity	FNR
CK 19 group 1	90%	72.4%	10%
CK 19 group 2	62.5%	100%	37.5%
MUC 1 group 1	62.5%	55%	37.5%
MUC 1 group 2	91.3%	29.7%	8.7%
CEA group 1	12.5%	71.7%	87.5%
CEA group 2	26.1%	89.2%	73.9%

Table 23: Sensitivity, specificity and false negative rates for CK 19, MUC 1 and CEA determined by RT PCR compared to H&E histology in group 1 and 2

FNR false negative rate

The best results were therefore achieved by CK 19 and MUC 1 however curiously MUC 1 expression was more sensitive in group 2 compared to group 1 which is contrary to what one would expect. CK 19 achieved a respectable sensitivity of 90% when half sentinel nodes were processed and a corresponding false negative rate of 10%. In both groups CEA proved to be a very poor breast cancer marker particularly in terms of sensitivity and false negative rate.

CK 19 expression agreed with the H&E histology most often in both group as shown in table 24 below. Despite the poor results given above, CEA expression concorded with H&E results in around two thirds of nodes in both groups. There were only just over a half of the nodes in both groups where MUC 1 expression and H&E histology were the same.

concordance with H&E histology	Group 1	Group 2
CK 19	72%	85%
MUC 1	55.9%	53.3%
CEA	64.7%	65%

Table 24: Comparison of marker expression by RT PCR with H&E histology in groups 1 and 2

When considering the upstaging potential of the marker expression results, group 1 was upstaged to a greater degree than group 2 for CK 19 and CEA, as one would expect however MUC 1 upstaged a greater percentage of nodes in group 2. MUC 1 produced the greatest upstaging rate in both groups of nodes as shown in table 25 below.

	Group 1 (n=68)	Group 2 (n=60)
CK 19 expression	36.8%	25%
CK 19 %upstaged	23.5%	0%
MUC 1 expression	47%	78.3%
MUC 1 %upstaged	39.7%	43.3%
CEA expression	26.5%	16.7%
CEA % upstaged	26.5%	6.7%

Table 25: Expression and upstaging rates of sentinel lymph nodes by CK 19, MUC 1 and CEA RT PCR

7.5. Concordance of marker expression

7.5.1. Group 1

The concordance between expression of CK 19 and MUC 1 was 72.1% with 49 out of 68 sentinel nodes being positive for both or negative for both. The remaining 19 sentinel nodes expressed either CK 19 or MUC 1.

Comparing expression rates of CK 19 and CEA, there was 51.5% concordance with 35 out of 68 sentinel nodes expressing either both or neither marker. The remaining 43 nodes expressed either CK 19 or CEA.

The concordance between MUC 1 and CEA expression was 44.1% with 30 out of 68 sentinel nodes displaying the same expression pattern for the 2 markers. The remaining 38 sentinel nodes expressed either MUC 1 or CEA.

The combined expression patterns were the same for all 3 markers in 23 out of the 68 nodes (33.8%) with 20 sentinel nodes expressing none and 3 expressing all 3. Of the nodes displaying no marker expression, there were 3 false negative cases where there were H&E positive non sentinel lymph nodes. In the other 17 nodes where no marker expression was found, all nodes were H&E negative. Of the nodes where all 3 markers were found to be positive, H&E histology was also positive in 1 and in the other 2 the sentinel node and all other axillary nodes were negative by H&E.

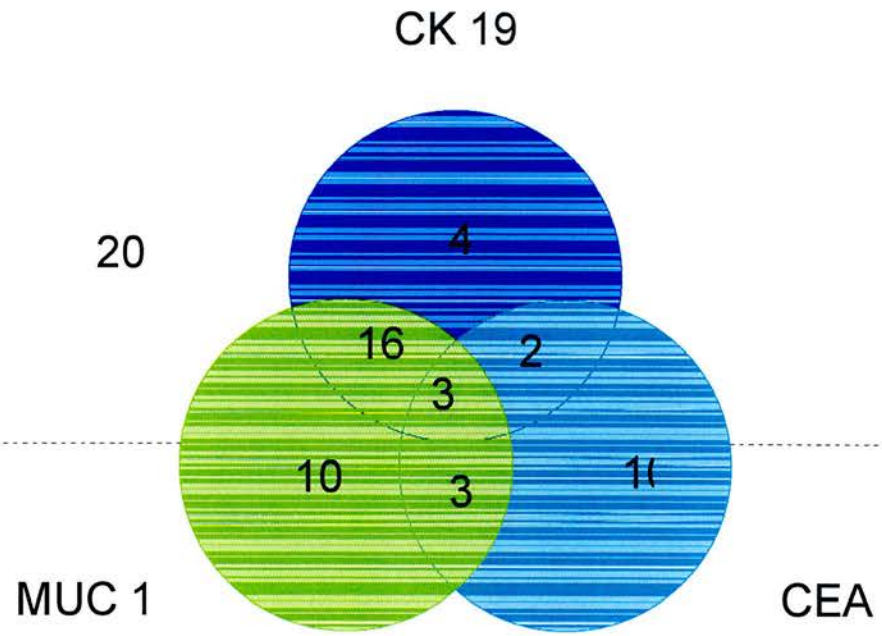


Figure 8: Venn diagram demonstrating marker expression by sentinel nodes from group 1 patients

(20 nodes displayed no expression)

7.5.2. Group 2

There was 46.7% concordance between CK 19 and MUC 1 expression, with 28 out of 60 nodes being positive for both markers or negative for both markers. The remaining 32 nodes all expressed MUC 1 but did not express CK 19. There were therefore no nodes which expressed CK 19 without MUC 1 expression.

Comparing expression of CK 19 and CEA, there was concordance in 71.7% (43/60) of sentinel nodes. In the remaining 17 nodes, some expressed CK 19 alone and some expressed CEA alone.

Comparing expression of MUC 1 and CEA, both nodes showed the same expression of both markers in 23 cases (38.3% concordance). In the remaining 37 nodes, all showed MUC 1 expression but no CEA expression. No nodes expressed CEA without MUC 1 expression.

Looking at the results for all 3 markers, there were only 17 out of 60 nodes (28.3%) displaying the same expression pattern. In 13 nodes there was no expression of any marker of which 9 were H&E negative and 4 were H&E positive. In 4 nodes, which were all H&E positive, all 3 markers were expressed. Of the 13 nodes negative for all 3 markers, 3 represented false negative nodes and so none of the PCR reactions had upstaged these sentinel nodes and 2 other sentinel nodes were H&E positive. The remaining 8 sentinel nodes with no marker expression were from patients in whom all nodes were H&E negative. Using the results of the 3 PCR reactions together therefore had incorrectly staged at least 5 patients (10%).

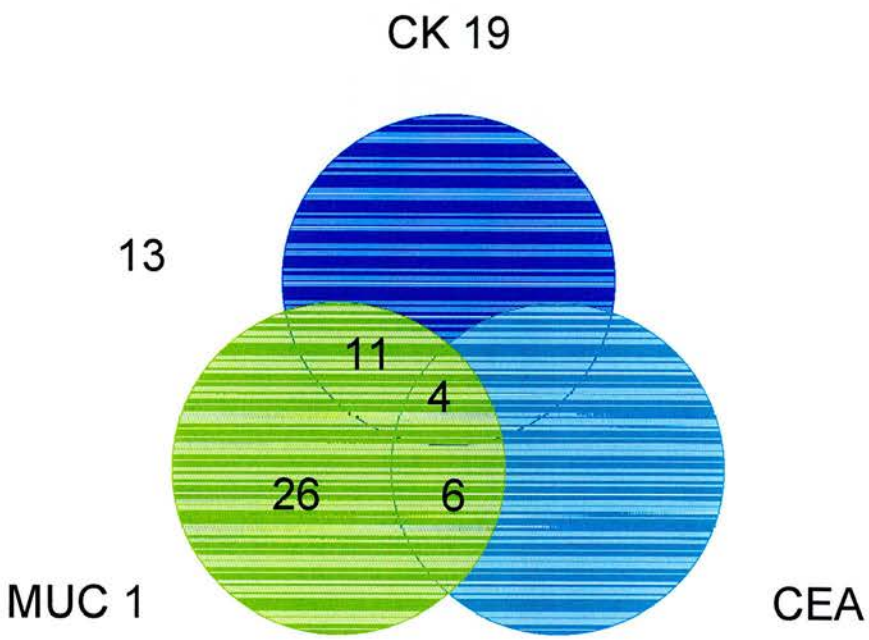


Figure 9: Venn diagram demonstrating marker expression by sentinel nodes from group 2 patients

(13 nodes displayed no marker expression)

7.6. Association of marker expression with Nottingham Prognostic Index

While the associations between the 3 markers and H&E histology were poor, inspection of the data indicated a strong association between CK 19 expression and the Nottingham Prognostic Index (NPI) values for both groups 1 and 2 (figure 10). In group 1, the mean NPI value of CK 19 positive nodes was 4.3 compared to a mean value of 3.4 in the CK 19 negative nodes ($p = 0.0046$; unpaired t-test) and in group 2, the mean NPI of the CK 19 positive nodes was 5.7 compared to only 4.0 in the CK 19 negative nodes ($p < 0.0001$; unpaired t-test). These data suggest that both tumour size and grade as well as the extent of lymph node involvement contribute to the likelihood of detection of CK 19 in the sentinel node.

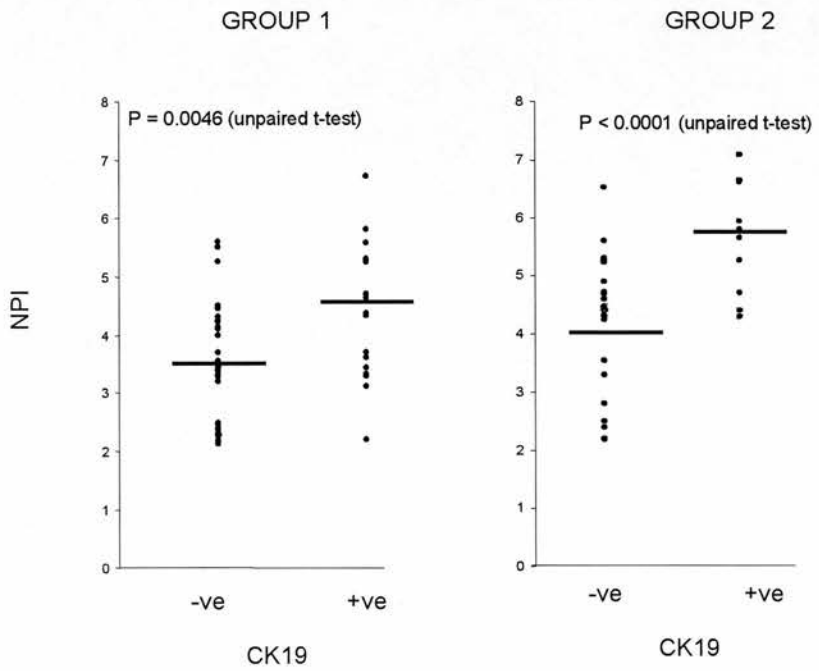


Figure 10: Association between CK 19 expression and NPI in groups 1 and 2

In contrast, the mean NPI values between patients with MUC 1 positive and MUC 1 negative sentinel nodes were not significantly different for either group 1 or 2 ($p = 0.21$ and 0.81 respectively) as was the case for CEA ($p = 0.12$ and 0.43 respectively).

Chapter 8
Discussion

Sentinel node biopsy has emerged as an alternative to axillary dissection and sampling in the staging of breast cancer however it is being performed by relatively few UK breast surgeons regularly. In this country it remains largely trial-based and progress lags behind many centres in the United States and Europe where the technique is now the standard of care for early breast cancer where studies with medium term follow up have shown no difference in regional recurrence or survival compared to axillary clearance.

One of the most important unanswered questions regarding the technique is how best to analyse the sentinel node. Serial sectioning and immunohistochemistry identify disease missed by standard H&E histology and RT PCR is a molecular technique which can identify individual cancer cells. The success of molecular analysis depends on the identification of a sensitive and specific breast cancer marker. So far such a marker or panel of markers has been elusive. There is conflicting evidence in the literature regarding the sensitivity and specificity of all the putative breast cancer markers described in lymph nodes of breast cancer patients and healthy controls. Until a reliable marker or panel of markers is identified RT PCR cannot replace histological assessment of sentinel nodes. At present therefore only part of any sentinel node can be processed for RT PCR analysis. If a sensitive and specific marker is identified then the clinical significance of disease detected in this way could be addressed by long term follow up studies.

Three putative breast cancer tumour antigens or 'markers' that have been previously studied are CK 19, MUC 1 and CEA. One study found 100% expression of CK 19 by primary breast tumours (Noguchi et al. 1996) however another study found that 10% of breast cancers were CK 19 negative (Papadimitriou et al. 1993). The expression of MUC 1 by breast cancer cells appears to be universal however CEA expression is variable with one study reporting 75% expression of CEA by primary breast tumours (Marchetti et al. 2001).

The aim of this thesis was to evaluate sentinel lymph nodes from breast cancer patients by RT PCR looking for the presence of CK 19, MUC 1 and CEA. In particular different

amounts of sentinel node tissue, half the node (from group 1 patients) versus a 4μm section (from group 2 patients), were processed for RT PCR. The RT PCR results were correlated with H&E histology and this allowed the upstaging potential of RT PCR to be compared in the 2 tissue groups. One hypothesis proposed at the start of the study was that the RT PCR results would correlate more closely with the H&E histology when the results of all three markers were considered together rather than any of these markers looked at in isolation. A discrepancy between H&E and RT PCR results was expected to some degree due to the fact that the 2 techniques were performed on different parts of each node. However RT PCR should be capable of detecting small disease foci in at least a proportion of the H&E positive nodes and combining the results for all 3 markers should maximise the correlation with H&E results. RT PCR should also hopefully up stage some additional H&E negative sentinel nodes. As the RT PCRs for the 3 markers were performed on the same tissue from each node, the expression rates of the 3 markers should correlate closely with each other in the presence of metastatic disease if these represent sensitive breast cancer markers. One would also expect higher expression rates of the 3 markers the greater the amount of tissue processed.

As expected, there was no marker which was expressed by all H&E positive sentinel nodes in either group. CK 19 expression was 90% sensitive in the sentinel nodes from group 1 with a corresponding false negative rate of 10%. The results for this marker were, as expected, poorer in group 2 where less tissue was processed (sensitivity 62.5%). MUC 1 expression was 91.3% sensitive in the group 2 sentinel nodes but less sensitive in group 1, contrary to that predicted. Expression of CEA was found to be very unreliable with sensitivities of only 12.5% and 26.1% in groups 1 and 2 respectively. The corresponding false negative rates were very high. One would have predicted that CEA would give the poorest results given that it is the least widely expressed of the three markers in breast cancers.

Comparing the expression rates of the different markers with each other, the results were different between the 2 groups. The pairing that agreed the most in group 1 was CK 19 and MUC 1 (72.1% concordance) and in group 2 was CK 19 and CEA (71.7%

concordance). In group 1 only about a third of the nodes (33.8%) displayed similar expression rates ie all 3 positive or all 3 negative. Importantly, 3 sentinel nodes that were false negative nodes based on H&E histology did not display expression of any of the 3 markers. In group 2, 28.3% of nodes were either positive or negative for all 3 markers. In this group there were also 3 false negative sentinel nodes that did not express any marker. Given that exactly the same tissue was examined for expression of the 3 markers, these figures confirm that the technique is unreliable in identifying metastatic disease with respect to these particular markers. As discussed already the sensitivity rate calculated for CEA would suggest that it is especially unreliable and therefore the poor correlations between CEA and CK 19 and between CEA and MUC 1 are probably due largely to CEA. CK 19 and MUC 1 expression rates agreed in nearly three quarters (72.1%) of group 1 nodes but in less than half (46.7%) of group 2 nodes.

Similar expression rates were found in groups 1 and 2 for each marker. This is contrary to one of the hypotheses set out at the start and is interesting given that the amount of tissue processed for RT PCR was much greater for group 1 and therefore one would have expected higher expression rates in this group. Group 2 contained a much higher proportion (50%) of histologically node positive patients compared to group 1 (18%) and this may explain why expression rates were not higher in group 1 compared to group 2. The upstaging rates of RT PCR however were greater for group 1 compared to group 2 for CK19 and CEA. For MUC 1 the upstaging rates were similar (39.7% versus 43.3%) in groups 1 and 2.

Obviously the main objective of any type of analysis of axillary nodes is to identify disease and therefore correctly stage the patient. It is therefore disappointing that a technique that is capable of identifying individual tumour cells missed 3 false negative sentinel nodes in both groups. There are various possible explanations for these false negatives. Firstly, this study would suggest that none of the 3 markers studied was 100% sensitive ie they were not expressed by breast cancer cells. This could have been further validated by either performing RT PCR for each marker on the primary tumours or by using immunohistochemistry to look for marker expression in the sentinel nodes.

Secondly, these cases may represent true cases of 'skip' metastases where the sentinel node genuinely does not contain the disease but other axillary nodes, higher up in the lymphatic chain, do harbour metastases. Thirdly, in some cases a sentinel node or nodes may have been missed and therefore represent failures of the technique itself.

The sensitivity of RT PCR in this study has been discussed. The specificity of any putative marker must also be considered in order that patients are not over staged by the technique. The specificity rates calculated in this study are the percentages of nodes that are H&E negative ('true negatives') that are correctly diagnosed by RT PCR. Clearly there will be some sentinel nodes designated as negative by the H&E histology performed which do harbour metastases. Some of these will have been upstaged by RT PCR. The definition of a 'true negative' sentinel node and a 'true positive' sentinel node is controversial but the only undisputed metastases of clinical significance are those detectable by H&E and so the specificities calculated above are relevant based on the literature to date. CK 19 expression reached 100% specificity in the sentinel nodes from group 2 however expression of the same marker was only 72.4% specific in relation to H&E histology in group 1. The specificities of CEA and MUC 1 in particular were disappointing.

In order to determine the true specificity of a potential marker in identification of axillary metastases, the expression rates in normal lymph nodes must be determined. Lymph nodes from healthy controls were not included in this study and the literature is confusing on this point. There are studies which have found both the presence and absence of CK 19 and MUC 1 in control lymph nodes as discussed above. It therefore is possible that some of the sentinel nodes that expressed the markers examined in this study represented 'false positives' due to expression by normal tissue in some individuals.

A significant association was found in both groups 1 and 2 between expression of CK 19 as determined by RT PCR and the NPI. This would suggest that patients with higher grade and or larger tumours may be more likely to express CK 19 in their sentinel nodes. The other 2 markers studied did not display a similar association.

Included in this study were all patients presenting to the clinic with a clinically node negative breast cancer and in fact the patients from group 2 included larger higher grade cancers than would ordinarily be suitable for sentinel node biopsy. There was a high proportion of node positive patients in this group – two thirds of the patients in whom a sentinel node was not detected and around half of the patients in whom a sentinel node was harvested. Clinical examination is notoriously inaccurate in the assessment of nodal status as discussed above and the use of preoperative axillary ultrasound with FNA or core biopsy of any abnormal looking nodes would have avoided so many node positive patients undergoing the technique at all. This approach has developed over the last few years and will increasingly be used to select patients for sentinel node biopsy. In 3 cases where no sentinel node was identified the axilla was found to be extensively diseased and this fits with the theory that blockage of lymphatic channels by tumour can hinder dissemination of blue dye and colloid into the axillary lymphatics. These tumour factors undoubtedly also contributed to the poor detection rate obtained.

All of the sentinel node biopsies carried out prospectively during this study for the group 2 patients were done using the combination of radioactive colloid with a pre-operative lymphoscintigram and intra-operative blue dye. In this group of 50 patients in whom a sentinel node was identified, we found that blue dye identified the sentinel node more often than the radioactive colloid. In 48 patients the sentinel nodes were blue and in 26 out of 50 patients the sentinel nodes were hot. There were only 5 out of 60 sentinel nodes that were hot and not blue. One study found that even following a positive scan, the addition of blue dye increased the detection rate and decreased the false negative rate (Degnim et al. 2005).

Intra-operative frozen section was performed on 103 sentinel lymph nodes with a false negative rate of 21.2%, as expressed on a node basis. The sensitivity of frozen section in identifying a positive sentinel node was 73%. This compares favourably with the literature where sensitivities of between 59% and 79% have been reported as described above. A positive frozen section allows axillary disease to be accurately diagnosed at the time of surgery and therefore completion axillary surgery carried out at the same

operation. A false negative result will subject some patients to a second procedure however nothing has been lost by carrying out a frozen section. The average time taken for the frozen section to be reported was 25 minutes and during this time the wide local excision or mastectomy was carried out.

In conclusion, this study found that none of the 3 markers examined was sensitive or specific enough to diagnose sentinel node metastases in breast cancer patients in clinical practice. There was poor correlation between conventional H&E histology and the expression of CK 19, MUC 1 and CEA and more importantly poor correlation between the expression of the individual markers suggesting that there was more to these discrepancies than just sampling error. Contrary to one of the hypotheses of this study, the expression of CK 19, MUC 1 and CEA did not depend on the amount of tissue processed for RT PCR in this study however the upstaging rate was greater in the group where half the node was processed compared to a 4μm section. All of the other hypotheses have been proved.

The significant correlation that was found between CK 19 expression by the sentinel nodes from both groups and NPI suggests that factors other than axillary node status, ie tumour grade and or size, may contribute to the likelihood of CK 19 expression by the sentinel node. Further work should therefore be done on this marker in particular to further explore this association. Other markers may in the future prove to be more reliable in determining the presence of sentinel node metastases. The clinical significance of any such marker expression as detected by RT PCR could then be evaluated by a long term follow up study.

Bibliography

Aitken RJ, Gaze MN et al (1989). Arm morbidity within a trial of mastectomy and either node sample with selective radiotherapy or axillary clearance. Br. J. Surg. 7(6): 568-571

Al-Shibli KI, Mohammed HA et al (2005). Sentinel lymph nodes and breast cancer: analysis of 70 cases by frozen section. Ann. Saudi Med. **25**(2): 111-114

Albertini JJ, Lyman GH et al (1997). Lymphatic mapping and sentinel node biopsy in patients with breast cancer. JAMA **277**(10): 791-792

Albertini JJ et al (1996). Intra-operative radiolymphoscintigraphy improves sentinel lymph node identification for patients with melanoma. Ann. Surg. **223**: 17

Alex J et al (1993). Gamma probe guided lymph node localization in malignant melanoma. Surg. Oncol. **2**: 303

Anan K, Mitsuyama S et al (2000). Axillary lymph node metastases in patients with small carcinomas of the breast: is accurate prediction possible. Eur. J. Surg. **166**: 610-615

Attiyeh FF, Jensen M et al (1977). Axillary micrometastases and macrometastases in carcinoma of the breast. Surg. Gynecol. Obstet. **144**: 839-842

Axelsson CK, Mouridsen HT et al (1992). Axillary dissection of level I and II lymph nodes is important in breast cancer classification. Eur.J. Surg. **28A**: 1415-1418

Axelsson CK, Mouridsen HT et al (2007). Axillary staging during surgery for breast cancer. Br. J. Surg. Jan 2007 (epub ahead of print)

Balslev I, Axelsson CK et al (1994). The NPI applied to 9144 patients from the studies of the Danish breast cancer cooperative group. Br. Cancer. Res. Treatment **32**(3): 281-290

Bass SS, Lyman GH et al (1999). Lymphatic mapping and sentinel lymph node biopsy. Breast J. **5**(5): 288-295

Bartek J, Taylor-Papadimitriou J et al (1985). Patterns of expression of keratin 19 as detected with monoclonal antibodies in human breast tissues and tumours. Int. J. Cancer **36**(6): 299-306

Baxter N et al (1996). Clinical behaviour of untreated axillary nodes after local treatment for primary breast cancer. Ann. Surg. Oncol. **3**: 235-240

Borgstein PJ, Meijer S et al (1997). Intradermal blue dye to identify sentinel lymph node in breast cancer. Lancet **349**: 1668-1669

Borgstein PJ, Pijpers R et al (1998). Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. J. Am. Coll. Surg. **186**: 275-283

Bostick PJ, Chatterjee S et al (1998). Limitations of specific reverse transcription polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. J. Clin. Oncol. **16**: 2632-2640

Bottini A, Berruti A et al (1997). Influence of neoadjuvant chemotherapy on serum tumour markers CA 15.3, MCA, CEA, TPS and TPA in breast cancer patients with operable disease. Tumour Biol. **18**(5): 301-310

Bourgeois P, Nogaret JM et al (2003). How 'hot' is the pathologically positive sentinel lymph node in breast cancer patients. Nucl. Med. Commun. **24**(5): 513-518

Branagan G, Hughes D et al (2002). Detection of micrometastases in lymph nodes from patients with breast cancer. Br. J. Surg. **89**: 86-89

Brogi E, Torres-Matundan E et al (2005). The results of frozen section, touch preparation and cytological smear are comparable for intraoperative examination of sentinel lymph nodes – a study of 133 breast cancer patients. Ann. Surg. Oncol. **12**(2): 173-180

Busch RM et al (1963). Roentgenographic visualisation of human testicular lymphatics; a preliminary report. J. Urol. **89**: 106-110

Byrne J, Walron R et al (1987). The use of monoclonal antibodies for the histopathological detection of mammary axillary micrometastases. Eur. J. Surg. **13**: 409-411

Cabanas RM (1977). An approach for the treatment of penile carcinoma. Cancer **39**: 456

Carter CL, Allen C et al (1989). Relation of tumour size, lymph node status and survival in 24 740 breast cancer cases. Cancer **63**: 181-187

Chadha M, Chabon AB et al (1994). Predictors of axillary lymph node metastases in patients with T1 breast cancer. A multivariate analysis. Cancer **73**(2): 350-353

Chagpar A, Martin RC 3rd et al (2004). Validation of subareolar and periatreolar injection techniques for breast sentinel lymph node biopsy. Arch. Surg. **139**(6): 614-618

Chagpar A, Middleton LP et al (2005). Clinical outcome of patients with lymph node negative breast cancer who have sentinel lymph node micrometastases detected by IHC. Cancer **103**(8): 1581-1586

Chan HY, Behen S et al (1993). Adequacy of axillary node sampling for evaluation of the axilla and control of axillary disease in breast cancer. Eur. J. Surg. **19**: 213-214

Chen Z-L, Wen D-R et al (1991). Occult metastases in the axillary lymph nodes of patients with breast cancer node negative by clinical and histological examination and conventional histology. Dis. Markers **9**: 239-248

Chetty U (2001). Axillary node sample to evaluate the axilla. World J. Surg. **25**: 73-779

Clarke D, Khonji NI et al (2001). Sentinel node biopsy in breast cancer: ALMANAC trial. World J. Surg. **25**: 819-822

Clarke D, Newcombe RG et al (2004). The learning curve in sentinel node biopsy: the ALMANAC experience. Ann. Surg. Oncol. **11**(3suppl): 211S-215S

Coveney EC, Geraghty JG et al (1995). The clinical value of CEA and CA 15.3 in breast cnacer management. Int. J. Biol. Markers **10**(1): 35-41

Cox CE, Pendas S et al (1998). Guidelines for sentinel node biopsy and lymphatic mapping of patients with breast cancer. Ann. Surg. **227**: 645-653

Cserni G (1999). The reliabililty of sampling 3 to 6 nodes for staging breast cancer. J. Clin. Pathol. **52**(9): 681-683

Cserni G, Amendoeira I et al (2004). Discrepancies in current practice of pathological evaluation of sentinel lymph nodes in breast cancer. Results of a questionnaire based survey by the European Working Group for Breast Screening Pathology. J. Clin. Path. **57**(7): 695-701

Datta YH, Adams PT et al (1994). Sensitive detection of occult breast cancer by RT PCR. J. Clin. Oncol. **12**(3): 475-482

deCremoux P, Extra JM et al (2000). Detection of MUC 1-expressing mammary cancer cells in the peripheral blood of breast cancer patients by real time PCR. Clin. Cancer Res. **6**(8): 3117-3122

Degnim AC, Oh K et al (2005). Is blue dye indicated for sentinel lymph node bio-psy in breast cancer patients with a positive lymphoscintigram. Ann. Surg. Oncol. **12**(9): 686-688

D'Eredita G, Giardina C et al (2001). Prognostic factors in breast cancer: the predictive value of the NPI in patients with long term follow up that were treated in a single institution. Eur. J. Cancer **37**(5): 591-596

Dell'Orto P, Biasi MO et al (2006). Assessing the status of axillary sentinel lymph nodes of breast carcinoma patients by a real-time quantitative RT-PCR assay for mammaglobin 1 mRNA. Breast Cancer Res.Treat. **98**(2): 185-190

deMascarel I, Bonichon F et al (1992). Prognostic significance of breast cancer axillary lymph node micrometastases assessed by two special techniques: reevaluation with longer follow up. Br. J. Cancer **66**: 523-527

Dent DM (1996). Axillary lymphadenectomy for breast cancer. Arch. Surg. **131**: 1125-1127

Early breast cancer trialists collaborative group; effects of radiotherapy and surgery in early breast cancer; an overview of randomised trials (1995). NEJM **333**: 1444

Ebeling FC, Schmitt UM et al (1999). Tumour markers CEA and CA 15.3 as prognostic factors in breast cancer – univariate and multivariate analysis. Anticancer Res. **19**(4A): 2545-2550

Eltahir EM, Mallinson DS et al (1998). Putative markers for the detection of breast carcinoma cells in blood. Br. J. Cancer **77**(8): 1203-1207

Eubus D, Leitch AM et al (1999). Hot, blue or hot and blue: maximising the accuracy of mammary sentinel lymph node biopsy (abstract). Presented at the 52nd annual meeting of the society of surgical oncology. Orlando, FL

Fabisiewicz A, Kulik J et al (2004). Detection of circulating breast cancer cells in peripheral blood by a 2 marker RT PCR assay. Acta. Biochim Pol. **51**(3): 747-755

Fisher B, Fisher ER et al (1966). Transmigration of lymph nodes by tumour cells. Science **152**: 1397-1398

Fisher B, Bauer M et al (1985). NSABP B-06 trial – 5 year results of a randomised controlled trial comparing total mastectomy with or without radiation in the treatment of breast cancer. NEJM **312**: 665-673

Fisher B, Redmond C et al (1985). Ten year results of a randomised clinical trial composing radical mastectomy and total mastectomy with or without radiation. NEJM **312**: 674-681

Fisher B, Redmond C et al (1989). Eight year results of a randomised clinical trial comparing total mastectomy and lumpectomy with or without irradiation in the treatment of breast cancer. NEJM **320**: 822

Fisher ER, Swamidoss S et al (1978). Detection and significance of occult axillary node metastases in patients with invasive breast cancer. Cancer **42**: 2025-2031

Fleissig A, Fallowfield LJ et al (2006). Post-operative arm morbidity and quality of life. Results of the ALMANAC trial comparing sentinel node biopsy with standard axillary treatment in the management of patients with early breast cancer. Br. Ca. Res. Treatment **95**(3): 279-293

Flett M, Going JJ et al (1998). Sentinel node localization in patients with breast cancer. Br. J. Surg. **857**: 991-993

Freneaux P, Nos C et al (2002). Histological detection of minimal metastatic involvement in axillary sentinel nodes: a rational basis for a sensitive methodology usable in daily practice. Mod. Pathol. **15**(6): 641-616

Frisell J, Bergqvist L et al (2001). Sentinel node in breast cancer – a Swedish pilot study of 75 patients. Eur. J. Surg. **167**: 179-183

Forrest APM et al (1995). The Edinburgh randomised trial of axillary sampling or clearance after mastectomy. Br. J. Surg. **82**: 1504-1508

Fournier K, Schiller A et al (2004). Micrometastases in the sentinel lymph node of breast cancer does not mandate completion axillary dissection. Ann. Surg. **239**(6): 859-863

Gaglia P, Bussone R et al (1987). The correlation between the spread of metastases by level in the axillary nodes and disease free survival in breast cancer. A multifactorial analysis. Eur. J. Cancer Clin. Oncol. **23**(6): 849-854

Galea MH, Blamey RW et al (1993). The Nottingham Prognostic Index in primary breast cancer. Br. Cancer. Res. Treatment **25**(3): 283

Gaston MS, Dixon JM (2004). A survey of surgical management of the axilla in UK breast cancer patients. Eur. J. Cancer **40**(11): 1738-1742

Gill PG (2004). Sentinel lymph node biopsy versus axillary clearance in operable breast cancer: The RACS SNAC trial. Ann. Surg. Oncol. **11**(3suppl): 216S-221S

Gillanders WE, Mikhitarian K et al (2004). Molecular detection of micrometastatic breast cancer in histopathogy negative axillary lymph nodes correlates with traditional predictors of prognosis: an interim analysis of a prospective multi-institutional cohort study. Ann. Surgery **239**(6): 828-837

Gimmi CD, Morrison BW et al (1996). Breast cancer associated antigen, DF3/MUC 1, induces apoptosis of activated human T cells. Nat. Med. **2**(12): 1367-1370

Giuliano AE, Kirgan DM et al (1994). Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann. Surg. **220**: 391-401

Given M, Scott M et al (2000). The predictive value of tumour markers CA 15.3, TPS and CEA in breast cancer recurrence. Breast **9**(5): 277-280

Grabau DA, Rank F et al (2005). Intraoperative frozen section examination of axillary lymph nodes in breast cancer. APMIS **113**(1): 7-12

Graverson HP et al (1988). Risks of axillary recurrence in node-negative patients following partial dissection of the axilla. Eur. J. Surg. Oncol **14**: 407-412

Guadagni F, Ferroni P et al (2001). A re-evaluation of CEA as a serum marker for breast cancer : a prospective longitudinal study. Clin. Cancer Res. **7**(8): 2357-2362

Haid A, Knauer M et al (2006). Medium-term follow-up data after sentinel node biopsy alone for breast cancer. Eur. J. Surg. Oncol. **32**(10): 1180-1185

Hainsworth PJ, Tjandra JJ et al (1993). Detection and significance of occult metastases in node-negative breast cancer. Br. J. Surg. **80**: 459-463

Halsted WS (1907). The results of radical operations for the cure of carcinoma of the breast. Ann. Surg. **56**: 1-19

Halter SA, Fraker LD et al (1984). CEA expression and patient survival in cancer of the breast. Oncology **41**(5): 297-302

Heatley M, Maxwell P et al (1995). Cytokeratin intermediate filament expression in benign and malignant breast disease. J. Clin. Oncol. **48**(1): 26-32

Hill ADK (1999) Lessons learned from 500 cases of lymphatic mapping for breast cancer. Ann. Surg. **229**: 528-535

Huvos AG, Hutter RVP et al (1971). Significance of axillary macrometastases and micrometastases in breast cancer. Ann. Surg. **173**: 44-46

Inokuchi M, Ninomiya I et al (2003). Quantitative evaluation of metastases in axillary lymph nodes of breast cancer. Br. J. Cancer **89**(9): 1750-1756

International (Ludwig) Breast Cancer Study Group (1990). Prognostic importance of occult axillary lymph node micrometastases from breast cancers. Lancet **2**: 1565-1568

Issacs RJ, Ford JM et al (1993). Role of computed tomography in staging of breast cancer. Br. J. Surg. **80**: 1137

Ivens D et al (1992). Assessment of morbidity from complete axillary dissection. Br. J. Cancer. **66**: 136-138

Iwase H, Kobayashi S et al (1995). Evaluation of serum tumour markers in patients with advanced or recurrent breast cancer. Br. Ca. Res. Treat. **33**(1); 83-88

Jezersek B, Cervek J et al (1996). Clinical evaluation of potential usefulness of CEA, CA 15.3 and MCA in follow up of breast cancer patients. Cancer Lett. **110**(1-2): 137-1344

Kahn HJ, Yang LY et al (2000). RT PCR amplification of cytokeratin 19 mRNA in the blood of breast cancer patients: correlation with established prognostic parameters. Breast Cancer Res. Treat. **60**(2): 143-151

Kell K, Varga G et al (1970). Direct lymphology of the breast. Lymphology **1**: 3-12

Kern KA (1999). Sentinel lymph node mapping in breast cancer using subareolar injection of blue dye. J. Am. Coll. Surg. **189**(6): 539-545

Klevesath MB, Bobrow LG et al (2005). The value of immunohistochemistry in sentinel lymph node histopathology in breast cancer. Br. J. Cancer **92**(12): 2201-2205

Koller M, Barsuk D et al (1998). Sentinel lymph node involvement – a predictor for axillary node status in breast cancer – has the time come? Eur. J. Surg. **24**: 166-168

Krag DN, Julian TB et al (2004). NSABP-32: phase III randomised trial comparing axillary resection with sentinel lymph node dissection: a description of the trial. Ann. Surg. Oncol. **11**(3suppl): 208S-210S

Krag D, Weaver D et al (1998). The sentinel node in breast cancer – a multicenter validation study. NEJM **339**: 941-946

Krag DN et al (1993). Surgical resection and radiolocalization of the sentinel node in breast cancer using a gamma probe. Surg. Oncol. **2**: 335

Krismann M, Todt B et al (1995). Low specificity of cytokeratin 19 RT PCR analyses for detection of haematogenous lung cancer dissemination. J. Clin. Oncol. **13**:2769-2775

Kuenen-Boumeester V, Menke-Pluymers M et al (2003). Ultrasound-guided FNA cytology of axillary lymph nodes in breast cancer patients. A pre-operative staging procedure. Eur. J. Cancer **39**(2): 170-174

Layeeque R, Henry-Tillman R et al (2003). Subareolar sentinel node biopsy for multiple breast cancers. Am. J. Surg. **186**(6): 730-735

Layeeque R, Kepple J et al (2004). Intraoperative subareolar radioisotope injection for immediate sentinel lymph node biopsy. Ann. Surg. **239**(6): 841-845

Lemos S, Dias M et al (2005). Detection of axillary metastases in breast cancer patients using ultrasound and colour Doppler combined with FNA cytology. Eur. J. Gynaecol. Oncol. **26**(2): 165-166

Lewis D, Reinhoff WF (1932). A study of operations for cure of carcinoma of the breast: John Hopkins Hospital 1889-1931. Ann. Surg. **95**: 336-400

Lin PP, Allison DC et al (1993). Impact of axillary lymph node dissection on the therapy of breast cancer patients. J. Clin. Oncol. **11**: 1536-1544

Liu LH, Siziopikou KP et al (2000). Evaluation of axillary sentinel lymph node biopsy by immunohistochemistry and multilevel sectioning in patients with breast cancer. Arch Pathol. Lab. Med. **124**(11): 1670-1673

Lokich JJ, Zamcheck N et al (1978). Sequential carcinoembryonic antigen levels in the therapy of metastatic breast cancer: a predictor and monitor of response and relapse. Ann. Intern. Med. **89**(6): 902-906

Lumanchi F, Brandes AA et al (1999). Long term follow up study in breast cancer patients using serum tumour markers CEA and CA 15.3. Anticancer Res. **19**(5C): 4485-4489

Lumachi F, Brandes AA et al (2000). Sensitivity of serum tumour markers CEA and CA 15.3 in breast cancer recurrences and correlation with different prognostic factors. Anti cancer Res. **20**(6C): 4751-4755

Lyman GH, Giuliano AE et al (2005). American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early stage breast cancer. J. Clin. Oncol. **23**(30): 7703-7720

MacKaren G et al (1992). The role of axillary dissection in early stage breast cancer. Cancer Invest. **10**: 461-470

McGee JM, Youmans R et al (1996). The value of axillary dissection in T1a breast cancer. Am. J. Surg. **172**: 501-504

McIntosh SA, Going JJ et al (1999). Therapeutic implications of the sentinel lymph node in breast cancer. Lancet **354**(9178): 570

McMasters KM, Wong SL et al (2000). Pre-operative lymphoscintigraphy for breast cancer does not improve ability to identify axillary sentinel lymph nodes. Ann. Surg. **231**(5): 724-731

Manhani AR, Manhani R et al (2001). Cytokeratin 19 expression by RT PCR in the peripheral blood of breast cancer patients correlates with response to chemotherapy. Breast Cancer Res. Treat. **66**(3): 249-254

Mansel RE, Fallowfield L et al (2006). Randomized multicentre trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC trial. J. Natl. Ca. Inst. **98**(9): 568-569

Mansour EG, Hastert M et al (1983). Tissue and plasma CEA in early breast cancer. A prognostic factor. Cancer **51**(7): 1243-1248

Manzotti M, Dell'Orto P et al (2001). Reverse transcription polymerase chain reaction assay for multiple mRNA markers in the detection of breast cancer metastases in sentinel lymph nodes. Int. J. Cancer **95**(5): 307-312

Marchetti A, Buttitta F et al (2001). mRNA markers of breast cancer nodal metastases: comparison between mammoglobin and CEA in 248 patients. J. Pathol. **195**(2): 186-190

Martinez-Trufero J, de Lobera AR et al (2005). Serum markers and prognosis in locally advanced breast cancer. Tumori **91**(5): 522-530

Mascagni, P (1787). Vasrum lymphaticorm corporis humani historia et ichnographia. Senis: P. Carli

Mathieson O, Carl J et al (1990). Axillary sampling and the risk of erroneous staging of breast cancer. An analysis of 960 consecutive patients. Acta Oncol. **29**(6): 721-725

Mincey BA, Bammer T et al (2001). Role of axillary node dissection in patients with T1a and T1b breast cancer. Ann. Surg. **136**: 779-782

Mir K, Edwards J et al (2002). The CAG trinucleotide repeat length in the androgen receptor does not predict the early onset of prostate cancer. BJU international **90**: 573-578

Mitchell ML (2005). Frozen section diagnosis for axillary sentinel lymph nodes: the first 6 years. Mod. Pathol. **18**(1): 58-61

Moll R, Franke WW et al (1082). The catalogue of human cytokeratins: patterns of expression in normal epithelia, tumours and cultured cells. Cell **31**: 11-24

Morton DL et al (1992). Technical details of intra-operative lymphatic mapping for early stage melanoma. Arch. Surg. **127**: 392

Morton DL, Wen DR et al (1989). Pathophysiology of regional lymph node metastases in early melanoma studied by intra-operative mapping of the cutaneous lymphatics (abstract) WHO second international conference on melanoma, Venice:131

Motomura K, Komoike Y et al (2002). Multiple sectioning and immunohistochemistry staining of sentinel nodes in patients with breast cancer. Br. J. Surg. **89**(9): 1032-1034

Murray KL, Lambah PA (2002). Occult lymph node metastases in patients with 'node negative' breast cancer treated with conservation surgery and axillary node sample and who subsequently developed axillary recurrence. Breast **11**(3): 249-251

Myers RE, Sutherland DJ et al (1987). Carcinoembryonic antigen in breast cancer. Cancer **42**(35): 1520-1526

Nemoto T, Vana J et al (1980). Management and survival of female breast cancers: results of a national survey by the American College of Surgeons. Cancer **45**: 2917-2924

Nieweg OE, Kim EE et al (1993). Positron emission tomography with fluorine-18-deoxyglucose in the detection and staging of breast cancer. Cancer **71**: 3920

Noguchi S, Aihara T et al (1994). The detection of breast cancer micrometastases in axillary lymph nodes by means of RT PCR. Cancer **74**(5): 1595-1600

Noguchi S et al, Aihara T et al (1996). Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcription polymerase chain reaction – comparison between MUC 1 mRNA and keratin 19 mRNA amplification. Am. J. Pathol. **148**(2):649-656

Nori J, Vanzi E et al (2007). Role of axillary ultrasound examination in the selection of breast cancer patients for sentinel node biopsy. Am. J. Surg. **193**(1): 16-20

O'Hanlon DM, Kerin MJ et al (1995). An evaluation of preoperative CA 15.3 measurement in primary breast carcinoma. Br. J. Cancer **71**(6): 1288-1291

Ooka M, Sakita I et al (2000). Selection of mRNA markers for detection of lymph node micrometastases in breast cancer patients. Oncol. Reports **7**(3): 561-566

Orr RK (1999). The impact of prophylactic axillary node dissection on breast cancer survival – a Bayesian meta-analysis. Ann. Surg. Oncol. **6**(1): 109-116

Papadimitriou JT, Berdichevsky F et al (1993). Human models of breast cancer. Cancer Survival **16**: 59-69

Paraonkar AS, Beissner RS (2003). Evaluation of immunohistochemistry and multiple-level sectioning in sentinel lymph nodes from patients with breast cancer. Arch. Pathol. Lab. Med. **127**(6): 701-705

Patey DH, Dyson WH (1948). Prognosis of carcinoma of the breast in relation to the type of operation performed. Br J. Cancer **2**: 7

Pavlista D, Eliska O (2005). Cutaneous and subcutaneous lymphatic drainage of the breast. Lymphology **38**(2): 92-102

Pectasides D, Pavlidis N et al (1996). Clinical value of CA 15.3, mucin-like carcinoma-associated antigen and CEA in monitoring early breast cancer patients. Am. J. Clin. Oncol. **19**(5): 459-464

Peley G, Sinkovics I et al (2004). Subareolar injection of radioactive colloid for sentinel lymph node identification in breast cancer patients. Am. Surg. **70**(7): 625-629

Peley G, Toth J et al (2001). Immunohistochemistry and RT PCR on sentinel lymph nodes can improve the accuracy of nodal staging in breast cancer patients. Int. J. Biol. Markers **16**(4): 227-232

Pickren JW (1961). Significance of occult metastases. A study of breast cancer. Cancer **14**: 1266-1271

Reitsamer R, Peintinger F et al (2004). 200 sentinel lymph node biopsies without axillary lymph node dissection – no axillary recurrences after a 3 year follow up. Br. J. Cancer **90**(8): 1551-1554

Rosen PP, Lesser ML et al (1983). Discontinuous or 'skip' metastases in breast cancer. Analysis of 1228 axillary dissections. Ann. Surg. **197**(3): 276-283

Roumen RM, Kuijt GP et al(2001). Treatment of 100 patients with sentinel node negative breast cancer without further axillary dissection. Br. J. Surg **88**(12): 1639-1643

Roumen RMH, Valkenburg JGM et al (1997). Lymphoscintigraphy and feasibility of sentinel node biopsy in 83 patients with primary breast cancer. Eur. J. Surg. Oncol. **23**: 495-502

Rutledge H, Davis J et al (2005). Sentinel node micrometastases in breast cancer may not be an indication for completion axillary dissection. Mod. Pathol. **18**(6): 762-768

Sacks NPM, Baum M (1993). Primary management of cancer of the breast. Lancet **342**: 1402-1408

Sakaguchi M, Virmani A et al (2003). Clinical relevance of RT PCR for the detection of axillary lymph node metastases in breast cancer. Ann. Surg. Oncol. **10**(2): 117-125

Sato K, Tamaki K et al (2002). Clinically useful detection criteria for sentinel nodes in patients with breast cancer using a radioisotope technique. Jpn. J. Clin. Oncol. **32**(10): 403-406

Schoenfeld A, Kruger KH et al (1997). The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using IHC and reverse transcription polymerase chain reaction for keratin 19. Eur. J. Cancer **33**(6): 854-861

Scholl SM, Balloul JM et al (2000). Recombinant vaccinia virus encoding human MUC 1 and IL 2 as immunotherapy in patients with breast cancer. J. Immunother. **23**(5): 570-580

Schrenk P, Hatzl-Griesenhofer et al (2001). Follow up of sentinel node negative breast cancer patients without axillary lymph node dissection. J. Surg. Oncol **77**(3): 165-170

Schrenk P, Konstantiniuk P et al (2005). Prediction of non sentinel lymph node status in breast cancer with a micrometastatic sentinel node. Br. J. Surg. **92**(6): 707-713

Schroder CP, Ruiters MH et al (2003). Detection of micrometastatic breast cancer by means of real time quatitative RT PCR and immunostaining in perioperative blood samples and sentinel nodes. Int. J. Cancer **106**(4): 611-618

Sedmak DD, Meineke TA et al (1989). Prognostic significance of cytokeratin-positive breast cancer metastases. Modern Pathol. **2**: 516-520

Seidman JD, Schnaper LA et al (1995). Relationship of the size of the invasive component of the primary breast carcinoma to axillary lymph node metastasis. Cancer **75**: 65-71

Smillie T, Hayashi A et al (2001). Evaluation of feasibility and accuracy of sentinel node biopsy in early breast cancer. Am.J. Surg **181**: 427-430

Spittle MF (1995). Brachial plexus neuropathy after radiotherapy for breast cancer – lower doses and surgical management of the axilla may be the answer. BMJ **311**: 1516-1517

Steele RJC, Forrest APM (1985). The adequacy of lower axillary node sampling in obtaining lymph node status in breast cancer: a controlled randomised trial. Br. J. Surg. **72**: 368-369

Stewart FW, Treves N (1948). Lymphangiosarcoma in postmastectomy oedema. Cancer **1**: 64-81

Stiles HJ (1892). Contributions to the surgical anatomy of the breast and axillary lymphatic glands. Edin. Med. J. **37**: 1099

Sutterlin M, Bussen S et al (1999). Predictive value of CEA and CA 15.3 in the follow up of invasive breast cancer. Anticancer Res. **19**(4A): 2567-2570

Tadych K, Donegan WL et al (1987). Postmastectomy seromas and wound drainage. Surg. Gynecol. Obstet. **165**: 483-487

Tafra L, Lannin DR et al (2001). Multicenter trial of sentinel node biopsy for breast cancer using both technetium sulphur colloid and isosulfan blue dye. Ann. Surg. **233**: 51-59

Takei H, Suemasu K (2002). Sentinel lymph node biopsy without axillary dissection after an intrs-operative negative histological investigation in 358 invasive breast cancer cases. Breast Cancer **9**(4): 344-348

Takei H, Suemasu K et al (2006). Sentinel lymph node biopsy alone has no adverse impact on the survival of patients with breast cancer. Breast J. **12**(5 suppl 2): S157-164

Takei H, Suemasu K et al (2007). Recurrence after sentinel lymph node biopsy with or without axillary lymph node dissection in patients with breast cancer. Breast Cancer **14**(1): 16-24

Tanis PJ, Boom RPA et al (2001). Frozen section investigation of the sentinel node in malignant melanoma and breast cancer. Ann. Surg. Pathol. **8**: 222-226

Tate JJT, Lewis V et al (1989). Ultrasound detection of axillary lymph node metastases in breast cancer. Eur. J. Surg. Oncol. **15**: 139

Tew K, Irwig L et al. (2005). Meta-analysis of sentinel node imprint cytology in breast cancer. Br. J. Surg. **92**(9): 1068-1080

Topal U, Punar S et al (2005). Role of ultrasound-guided core needle biopsy of axillary lymph nodes in the initial staging of breast cancer. Eur. J. Radiol. **56**(3): 382-385

Torrenga H, Fabry H et al (2004). Omitting axillary lymph node dissection in sentinel node negative breast cancer patients is safe: a long term follow up analysis. J.Durg. Oncol. **88**(1): 4-7

Traweek, ST, Liu J et al (1993). Keratin gene expression in non-epithelial tissues. Detection with polymerase chain reaction. Am. J. Pathol. **142**: 1111-1118

Trojani M, deMascarel I et al (1987). Micrometastases to axillary lymph nodes from carcinoma of breast: detection by IHC and prognostic significance. Br. J. Cancer **55**: 303-306

Urban JA, Marjori MA (1971). Significance of internal mammary lymph nodes in breast cancer. Am. J. Roentgenol. **111**: 130-136

Van der Ent FWC, Kengen RAM et al (1993). Sentinel node biopsy in 70 unselected patients with breast cancer: increased feasibility by using 10mCi radiocolloid in combination with blue dye tracer. Surg. Oncol. **2**: 335

Van Dougan JA, Bartelink H et al (1992). EORTC 10801 trial – randomised controlled trial to assess the value of breast conservation therapy in stage I and II breast cancer. Monogr. Natl. Ca. Inst. **11**: 15-18

Van Lancker M, Goor C et al (1995). Patterns of axillary lymph node metastases in breast cancer. Am. J. Clin. Oncol. **18**(3): 267-272

Veronesi U et al (1971). Distribution of axillary node metastases in breast cancer. Am. J. Roentgenol **111**: 130-136

Veronesi U, Galimberti V et al (2005). Sentinel node biopsy in breast cancer: early results in 953 patients with negative sentinel node biopsy and no axillary dissection. Eur. J. Cancer **41**(2): 197-198

Veronesi U, Paganelli G et al (2006). Sentinel lymph node biopsy as a staging procedure in breast cancer: update of a randomised controlled study. Lancet Oncol. **7**(12): 983-990

Veronesi U, Saccozzi R et al (1981). Comparing radical mastectomy with quadrantectomy, axillary dissection and radiotherapy in patients with small cancers of the breast. NEJM **305**: 6

Veronesi U, Paganelli G et al (1997). Sentinel node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph nodes. Lancet **349**: 1864-1867

Viale G, Maiorano E et al (2001). Histologic detection and clinical implications of micrometastases in axillary sentinel lymph nodes for patients with breast cancer. Cancer **92**(6): 1378-1384

Virchow R (1849). Zur diagnose der Krebse in unterleibe. Die Medinische Reform **45**: 248

Weigelt B, Verduijn P et al (2004). Detection of metastases in sentinel lymph nodes of breast cancer patients by multiple mRNA markers. Br. J. Cancer **90**(8): 1531-1537

Wells CA, Heryet A et al (1984). The immunocytochemical detection of axillary micrometastases in breast cancer. Br. J. Cancer **50**: 193-197

Werner RS et al (1991). Arm oedema in conservatively managed breast cancer: obesity is a major predictive factor. Radiology **180**: 177-184

White RL Jr, Wilke LG (2004). Update on the NSABP and ACOSOG breast cancer sentinel node trials. Ann. Surg. **70**(5): 420-424

Wilkinson EJ, Hause LL et al (1982). Occult axillary lymph node metastases in invasive breast carcinoma: characteristics of the primary tumour and significance of the metastases. Pathology Annual **17**: 67-91

Wong IH, Yeo W et al (2001). Quantitative correlation of CK 19 mRNA level in peripheral blood with disease stage and metastasis in breast cancer patients: potential prognostic implications. Int. J. Oncol. **18**(3): 633-638

Xenedis N, Perraki M et al (2006). Predictive and prognostic value of peripheral blood cytokeratin 19 mRNA positive cells detected by real time PCR in node negative breast cancer patients. J. Clin. Oncol (epub ahead of print)

Yared MA, Middleton LP et al (2002). Recommendations for sentinel lymph node processing in breast cancer. Am. J. Surg. Pathol. **26**(3): 383-385

Zavagno G, Carcoforo P et al (2005). Axillary recurrence after negative sentinel lymph node biopsy without axillary dissection: a study on 479 breast cancer patients. Eur. J. Oncol. **31**(7): 715-720

Zhang Y, Ma QY et al (2006). Quantitative molecular diagnosis of axillary drainage fluid for prediction of locoregional failure in patients with 1 to 3 positive axillary nodes after mastectomy without adjuvant radiotherapy. Int. J. Radiat. Oncol. Bio. Phys. **64**(2): 505-511

Zogakis TG, Wetherille RE et al (2005). Intraoperative subareolar injection of 99mTc-labelled sulphur colloid results in consistent sentinel lymph node identification. Ann. Surg. Oncol. **12**(2): 167-172

Zotter S, Hageman C et al (1988). Tissue and tumour distribution of human polymorphic epithelial mucin. Cancer Res. **11**: 55-101

Appendix I – Patient information sheet

Royal Infirmary
16 Alexandra Parade
Glasgow G31 2ER

Switchboard: 0141 211 4000
Direct Dial:
Fax Number:

Patient Information Sheet

Sentinel Lymph Node biopsy in Patients with Breast Cancer.

We are inviting you to participate in a research study involving Glasgow Royal Infirmary and The Western Infirmary. It is important that you understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and do not hesitate to ask us about anything that you are not clear about.

What is this study for?

Breast cancer is known to spread from the breast to the lymph glands under the arm. At present patients diagnosed with breast cancer usually have these glands removed at the time of the surgery to remove the tumour itself. The glands are then examined by a pathologist who determines whether the cancer has spread to these glands and this is important in deciding whether further treatment such as chemotherapy is necessary. It has been demonstrated that cancer spreads to one gland (the sentinel node) first and that if this is free from tumour then the other glands should also be free. In the future we may be able to remove only the sentinel node if there is no sign of tumour spread in this gland. This will avoid the possible side effects that can occur when all the glands are removed such as arm swelling (lymphoedema). If you agree to participate in this study this will not mean that you will obtain these benefits as we will still be removing all of the glands from under your arm, as is current standard practice, after identifying the sentinel node.

What do I do if I take part?

If you agree to take part you will be asked to sign a consent form. On the afternoon before your surgery you will have an injection into your breast, around the tumour. This will involve a small dose of radioactivity (the maximum dose given to you will be 40MBq which is one hundred times lower than the dose used in some medical investigations such as lung and bone scans). A short time after the injection you will have a 'scan' which is completely painless and aims to detect glands under the arm to which the radioactive injection has spread – these 'hot spots' will be marked on the skin. When you are under anaesthetic for your surgery some blue dye (not radioactive) will be injected in a similar way and we will then use the radioactivity and the dye to detect the sentinel lymph node. This node will then be removed and sent to the pathologists for analysis. The surgery will then proceed in the standard way – ie the 'lump' or breast will be removed (as discussed with you before surgery) and the remaining glands from under the arm will also be removed.

IT SHOULD BE EMPHASISED THAT YOUR TAKING PART IS ENTIRELY VOLUNTARY, AND WHETHER YOU TAKE PART OR NOT WILL IN NO WAY AFFECT YOUR CURRENT OR FUTURE TREATMENT.

What will happen to the sentinel node?

After the sentinel node has been removed, it will be examined using various techniques which are designed to pick up very small traces of tumour spread. Some of these techniques are currently not done routinely on lymph glands that are removed from patients as this is not feasible with so many glands to examine. The sentinel node will also undergo the routine pathology tests that are currently standard practice. The other glands that are removed will also undergo standard examination.

Some tissue from your breast and from the sentinel and other lymph nodes will be kept frozen in the hospital, AS IS DONE ROUTINELY FOR EVERY PATIENT AT THE MOMENT.

What are the disadvantages and risks of taking part?

The only possible side effects are some pain associated with the first injection, done while you are awake (the radioactivity) and slight temporary discolouration of your skin and urine (for about 12 hours) – this is completely harmless. The detection and removal of the sentinel node adds about 15 minutes onto the time of your operation. The steps described to do this are in addition to standard treatment and so your treatment will in no way be different from patients not participating in the study.

Will my taking part in this study be confidential?

Any information that is collected from you during this study will be kept strictly confidential.

- PLEASE UNDERSTAND THAT AT ANY TIME YOU CAN WITHDRAW YOUR NAME FROM THIS STUDY WITHOUT AFFECTING YOUR CARE.
- THIS RESEARCH STUDY HAS BEEN REVIEWED BY THE GLASGOW ROYAL INFIRMARY RESEARCH ETHICS COMMITTEE.
- THIS RESEARCH IS BEING CARRIED OUT IN THE DEPARTMENTS OF SURGERY AND PATHOLOGY AT GLASGOW ROYAL INFIRMARY. NO ONE INVOLVED IN THE RESEARCH GETS PAID ESPECIALLY TO DO THIS STUDY.
- SHOULD YOU WISH TO OBTAIN ANY FURTHER INFORMATION, PLEASE CONTACT MARGARET FALCONER AT GLASGOW ROYAL INFIRMARY, TELEPHONE (0141) 211 5440.

Thank you for reading this and taking part in the study.

Version no. 2, November 2000

Appendix II – Patient consent form

Royal Infirmary
16 Alexandra Parade
Glasgow G31 2ER

Switchboard: 0141 211 4000
Direct Dial:
Fax Number:

Study Number: 00SG011
Patient Identification Number for this trial:

CONSENT FORM

The use of multiple sectioning, immunohistochemistry and RT-PCR to detect metastases in the sentinel lymph nodes of patients with breast cancer

Name of researcher: Miss Margaret Falconer, Professor Tim Cooke, Miss J Doughty, Dr JJ Going

Please initial box

1. I confirm that I have read and understand the information sheet dated November 2000..... (version ..No. 2...) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

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Health Quality Service

Appendix III – Conference proceedings

1. Upstaging of sentinel nodes in breast cancer by cytokeratin 19 RT-PCR
ME MacLean, J Edwards, JJ Going, TG Cooke, JMS Bartlett

Poster presentation, 25th annual San Antonio Breast Cancer Symposium, Texas
December 2002

2. Use of reverse transcription PCR to detect cytokeratin 19 expression in sentinel nodes of patients with breast cancer.
ME MacLean, J Edwards, JJ Going, TG Cooke, JMS Bartlett

Oral presentation, 3rd European Breast Cancer Conference, Barcelona
March 2002

3. Intra-operative frozen section reliably predicts sentinel node status in patients with breast cancer.
ME MacLean, CR Wilson, MM Flett, JJ Going, JMS Bartlett, TG Cooke

Oral presentation, 24th annual San Antonio Breast Cancer Symposium, Texas
December 2001

4. Intra-operative frozen section reliably predicts sentinel node status in breast cancer
ME MacLean, CR Wilson, MM Flett, JJ Going, TG Cooke

Oral presentation, West of Scotland Surgical Society annual meeting
October 2001

5. Intra-operative frozen section reliably predicts sentinel node status in patients with breast cancer.

ME MacLean, JJ Going, CR Wilson, MM Flett, TG Cooke

Oral presentation, 7th Nottingham International Breast Cancer Conference, September 2001

6. Intra-operative frozen section reliably predicts sentinel node status in patients with primary breast cancer

ME MacLean, JJ Going, MM Flett, CR Wilson, TG Cooke

Oral presentation, Association of Surgeons of Great Britain and Ireland Annual Meeting, Birmingham
April 2001

Appendix IV - Publications

1. Intra-operative frozen section reliably predicts sentinel node status in patients with breast cancer.

MacLean ME, Wilson CR, Flett MM, Going JJ, Bartlett JMS, Cooke TG.

Breast Cancer Research and Treatment 2001; **69**(3): 217

(Abstract)

2. Intra-operative frozen section reliably predicts sentinel node status in patients with primary breast cancer

ME MacLean, JJ Going, MM Flett, CR Wilson, TG Cooke

British Journal of Surgery 2001; **88** (S1): 40

(Abstract)

3. Intra-operative frozen section reliably predicts sentinel node status in patients with breast cancer.

ME MacLean, JJ Going, CR Wilson, MM Flett, TG Cooke

European Journal of Cancer 2001; **37**(S5): 18

(Abstract)

4. Use of reverse transcription PCR to detect cytokeratin 19 expression in sentinel nodes of patients with breast cancer.

ME MacLean, J Edwards, JJ Going, TG Cooke, JMS Bartlett.

European Journal of Cancer 2002; **38** (S3): S156

(Abstract)